Remarks

Claims 1-16 are pending in the application. Claims 15 and 16 are under examination, claims 1-14 having been previously withdrawn from consideration. Claim 15 has been amended. Claim 16 has been cancelled. An element has been removed from claim 15, and the phrase "wherein said endorepellin protein consists of amino acid residues 3687 to 4391 of the perlecan protein," has been added. Amendment of claim 15 presents no new subject matter. Support for the amendment to claim 15 is found throughout the specification, particularly at page 2, lines 4-7, page 3, line 16, page 19, lines 12-13, and Fig. 1f. Claim 17 has been added. Claim 17 is supported throughout the specification as filed and does not comprise new subject matter (page 19, lines 11-24; Fig. 1f, Fig. 1g).

The paragraph comprising lines 5-25 of page 12 of the specification as filed has been deleted. The paragraph has been deleted because it contained a phrase referring to endorepellin as a protein approximately between 210 amino acids and 705 amino acids in size, which phrase was alleged by the Examiner to cause the term "endorepellin" to be indefinite. This amendment is discussed more fully below. The paragraph also described derivatives and analogs of endorepellin, which by way of current amendment are no longer claimed in claim 15.

Rejection of Claims 15 and 16 under 35 U.S.C. § 112, second paragraph

Claims 15 and 16 stand rejected as allegedly being indefinite. Claim 16 has been canceled, therefore the rejection as to claim 16 is now moot. The Examiner alleges at page 2 of the Office Action that, although the terms "fragments," derivatives," and "analogs," were known by the skilled artisan at the time of filing, one of skill in the art would not know the metes and bounds of the terms used in the claims. The Examiner also alleges that the specification defines the terms in a broad scope, wherein fragments, analogs, or derivatives are any amino acid sequences which are substantially similar to the amino acid sequence of endorepellin. Although not necessarily agreeing with the reasoning of the Examiner, Applicant has amended the claims to recite endorepellin, wherein endorepellin consists of amino acid residues 3687 to 4391 of perlecan, and fragments of endorepellin. The claims no

longer include derivatives or analogs of endorepellin. Applicant respectfully submits that the term "fragments" is definite and that the specification as filed does allow determination of the metes and bounds of the term "fragment" as claimed.

Applicant asserts that the term "fragments," as applied to endorepellin, is not indefinite because it is adequately described throughout the specification as filed. Applicant respectfully points out that the specification as filed also describes the preparation of multiple specific fragments of endorepellin (see Fig. 1 and Examples). For example, the specification provides data comparing the activity of the full length sequence of endorepellin, comprising amino acids 3687-4391 (domain V) of the perlecan sequence (Murdoch et al., 1992, J. Biol. Chem., 267:12:8544-8557; copy enclosed), to seven different fragments disclosed in the specification (Figures 1f, 1g). The full length sequence of perlecan, as well as the description of its domains, were known to those of skill in the art at the time the specification was filed (Murdoch et al., 1992, J. Biol. Chem., 267:12:8544-8557).

The disclosure further demonstrates that two of the fragments of endorepellin, Δ1 and Δ5, comprising fragments a.a. 3687-4181 and a.a. 3927-4181 of perlecan, respectively, possess endostatin binding activity (page 19, lines 11-24; Fig. 1f, Fig. 1g.). The entire gene and protein sequences for perlecan and its domain V (referred to in the specification as filed as endorepellin) are provided in Figure 2 of reference number 5 cited in the specification (Murdoch et al., 1992, J. Biol. Chem., 267:12:8544-8557).

The specific endorepellin fragment names and lengths disclosed in the specification are based on the sequence published in Murdoch et al. For example, each fragment disclosed in the specification is described in terms of length, based on the specific amino acid residue positions of its first and last amino acids in the native amino acid sequence of perlecan. All of the amino acid residues and their positions are described as part of the sequence information disclosed in Murdoch et al. Thus, sequence information for endorepellin and its fragments was either disclosed in the specification as filed or was available to those of ordinary skill in the art at the time the application was filed. Armed with this information and with specifically defined biologically active fragments of endorepellin as disclosed in the specification, one of skill in the art would understand what is meant by a "fragment" of endorepellin.

Other fragments are also characterized in the specification. For example, the invention also provides for a 25 kDa cleavage fragment of endorepellin (81 kDa) which is reactive with the anti-His6 antibody directed against endorepellin (page 24, lines 4-7).

Thus, Applicant respectfully submits that ample support is provided for use of the term "fragment," as applied to endorepellin, and that armed with the disclosure of the specification as filed, one of ordinary skill in the art would be able to determine the metes and bounds of the term "fragments" of endorepellin as described in the specification and recited in claim 15. Applicant requests withdrawal of the 35 U.S.C. § 112, second paragraph, rejection as applied to this term.

The Examiner also alleges at pages 3-4 of the Office Action that the term "endorepellin" is indefinite and cannot be determined because the amino acid sequence and length is not specifically disclosed in the specification. It is the opinion of the Examiner that the specification discloses that endorepellin is the carboxy terminus portion of perlecan or domain V of perlecan (citing page 2 of the application). The Examiner also alleges that Murdoch et al. (1992, J. Biol. Chem., 267:12:8544-8557) discloses domain V as being 705 amino acids in length. The Examiner further alleges that Mongiat et al (2003, J. Biol. Chem. 278:6:4238-4239) defines endorepellin as the protein consisting of the entire domain V of perlecan (amino acids 3687-4391) and that the specification defines "endorepellin" at page 12 as being between 210 and 705 amino acids in length. It is the Examiner's opinion that one of skill in the art would have a difficult time determining the true amino acid sequence represented by the term endorepellin, e.g., whether it is a 210 amino acid protein, a 704 amino acid protein or a protein that falls within the ranges of 210-705 amino acids.

The Examiner then states at page 4 that "It is noted for the record that the instantly claimed protein has not been defined by a sequence. As such the claimed protein is not defined and is considered an indefinite laboratory term." Applicant respectfully disagrees.

Applicant respectfully submits that there was adequate disclosure of the term "endorepellin" in the specification as filed, and that there was adequate information known to those of skill in the art at the time the specification was filed, to determine the metes and bounds of the term "endorepellin." Applicant further asserts that adequate sequence information was available to determine the metes and bounds of the term "endorepellin."

As discussed above, Figures 1f and 1g disclose endorepellin, as well as various peptide fragments of endorepellin and the locations of the fragments, based on residue number, in both endorepellin and perlecan. In fact, a description of domain V of perlecan, or endorepellin, comprising amino acids 3687-4391 of the perlecan sequence, e.g., a peptide 705 amino acids in length, is provided in both Figures 1f and 1g of the specification as filed. The Examiner admits at page 3 of the Office Action that the specification at page 2 defines endorepellin as the carboxy terminus of perlecan or domain V of perlecan and that Murdoch et al. discloses domain V as being 705 amino acids in length. Thus, the specific location of the endorepellin sequence is described, as is the size of that sequence. Furthermore, Figures 1f and 1g, describe the biologic activity of full length endorepellin, relative to fragments of endorepellin. For example, fragments a.a. 3687-4181 (fragment Δ1; 495 amino acids in length) and a.a. 3927-4181 (fragment Δ5; 255 amino acids in length) retain functional activity at levels comparable to the functional activity of full length endorepellin (a.a. 3687-4391; Figures 1f and 1g). Thus, one of ordinary skill in the art would understand that endorepellin as described in the specification is a peptide of 705 amino acids. Furthermore, the nucleic acid and amino acid sequences of perlecan were provided in Figure 2 of reference 5 cited in the specification (Murdoch et al., 1992, J. Biol. Chem., 267:12:8544-8557), and therefore, were known to those of ordinary skill in the art at the time the specification was filed.

Murdoch et al. also presented a molecular model of all the domains of perlecan in Figure 3 (1992, J. Biol. Chem., 267:12:8544-8557). In addition, Murdoch et al. described several characteristics of domain V, including that it is a 705 amino acid terminal module of perlecan (page 8550, column 2, to page 8551, column 1, last paragraph). The endorepellin peptide and fragments disclosed in the specification as filed are based on the sequence published by Murdoch et al.

Furthermore, although not necessarily agreeing with the reasoning of the Examiner, in order to expedite prosecution of the application, Applicant has amended the specification and claims to better ensure that one of skill in the art can determine the amino acid sequence of endorepellin. To that end, in the section entitled "Endorepellin, analogs, and fragments thereof" on page 12 of the specification, the phrase referring to endorepellin as "approximately between 210 amino acids and 705 amino acids in size" has been deleted.

The remainder of that paragraph on page 12 has also been deleted as it refers to analogs and derivatives of endorepellin, and analogs and derivatives of endorepellin are no longer recited in claim 15.

The description of endorepellin on page 12 of the specification is the only place in the specification where endorepellin is described as a protein that is approximately between 210 amino acids and 705 amino acids in size. All other references to endorepellin in the specification refer to the 705 amino acid fragment of perlecan consisting of amino acid residues 3687 to 4391 of perlecan. The faulty description of endorepellin which is being deleted from the specification was imprecise and actually referred to fragments of endorepellin and their size, and not to endorepellin itself. For example, the fragment comprising amino acid residues 4182-4391, is a 210 amino acid fragment of endorepellin, also referred to as Δ7 (see Figure 1f). The 210 amino acid fragment is the smallest fragment disclosed in the specification. The statement should have more clearly indicated that the size of an endorepellin fragment could be between the size of the disclosed 210 amino acid fragment and the 705 amino acid fragment of perlecan called endorepellin. In addition, Claim 16, which recited "wherein said endorepellin protein is between 210 and 705 amino acids in size" has been cancelled.

Based on the foregoing, Applicant respectfully submits that "endorepellin" is not indefinite. Armed with the information provided in the specification and with the information available in the prior art at the time the specification was filed, one of ordinary skill in the art would be able to determine the metes and bounds of "endorepellin."

Applicant respectfully requests reconsideration and withdrawal of the rejection of claim 15 under 35 U.S.C. 112, second paragraph, for indefiniteness.

Rejection of Claims 15 and 16 under 35 U.S.C. § 112, first paragraph, written description

Claims 15 and 16 stand rejected for lack of written description under 35 U.S.C. § 112, first paragraph. Claim 16 has been cancelled, therefore the rejection as to this claim is now moot. At page 4 of the office action, the Examiner asserts that although he stated in the previous office action that the written description requirement was met for an endorepellin protein that was between 210-705 amino acids in length and not specifically for any and all

fragments, analogs, or derivatives, he now presents a new argument. The Examiner now alleges that the written description has only set forth endorepellin proteins that are of 704 (full length), 494 (Δ1), 239 (Δ2), 106 (Δ3), 78 (Δ4), 254 (Δ5), 283 (Δ6), and 209 (Δ7) amino acids in length. The Examiner then asserts that the written description is not commensurate in scope to any and all endorepellin fragments, analogs, and derivatives.

At page 5 of the Office Action, the Examiner states that "the instant specification is missing sequence identification numbers that specifically identify the fragments claimed with an associated sequence." The Examiner asserts that one of skill in the art would not be able to adequately determine if the claimed sequences are different from the other sequences disclosed in the art. The Examiner then alleges that despite the fact that the endorepellin sequence was disclosed in the art, the specification has not demonstrated that the Applicant was indeed in possession of any and all fragments, analogs, or derivatives of endorepellin. The Examiner further alleges that the specification is devoid of analog and derivative disclosure. Lastly, the Examiner alleges that only the fragments Δ1 to Δ7 meet the requirements for written description. Applicant respectfully disagrees for the reasons discussed below.

First, as discussed above, claim 15 has been amended to recite fragments of endorepellin, but not analogs and derivatives. Thus, the rejection as to analogs and derivatives of endorepellin is now moot.

Applicant respectfully submits that the specification as filed provides adequate written description for endorepellin fragments. The above remarks regarding 35 U.S.C. § 112, second paragraph support for endorepellin fragments, apply to the written description requirement of 35 U.S.C. § 112, first paragraph as well.

Applicant also respectfully points out a mathematical error by the Examiner in determining the length of endorepellin. Full length endorepellin is 705 amino acids in length, not 704 amino acids as stated by the Examiner. The full length endorepellin fragment of perlecan is amino acid residues 3687 to 4391. Endorepellin includes the first and last residues listed, thus, it is 705 amino acids in length.

As outlined in MPEP § 2163, a description need only describe in detail that which is new or not conventional. See *Hybritech v. Monoclonal Antibodies*, 802 F.2d 1367, 1384,

231 USPQ 81, 94; *Fonar Corp. v. General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805.

Preliminarily, it is well-settled law that the written description requirement is viewed in light of the state of the art and skill of the practitioner at the time the application was filed. In *Vas-Cath, Inc. v. Mahurkar,* 19 USPQ2d 1111 (Fed. Cir. 1991), the Court of Appeals for the Federal Circuit traced the development of the written description requirement under 35 U.S.C. §112, first paragraph. The *Vas-Cath* Court, in a unanimous opinion, noted approvingly that in a written description analysis, "[t]he primary concern is factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure." *Vas-Cath*, 19 USPQ2d at 116 (quoting *In re Wertheim*, 191 USPQ 90, 96 (C.C.P.A. 1976)). After discussing the policy reasons underlying the requirement, the Court set forth the standard for the written description requirement:

> The purpose of the "written description" requirement is broader than to merely explain how to "make and use"; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention. . . . The test for sufficiency of support in a parent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter."

Vas-Cath, 19 USPQ2d at 1117 (emphasis added) (quoting *Ralston Purina Co. v. Far-Mar-Co., Inc.*, 227 USPQ 177, 179 (Fed. Cir. 1985)). *Accord University of California v. Eli Lilly & Co.*, 43 USPQ2d 1398 (Fed. Cir. 1997). Therefore, it is well-settled that the knowledge of those skilled in the art informs the written description inquiry.

In determining the sufficiency of support in a disclosure with respect to the written description requirement, "it is not necessary that the application describe the claimed invention in *ipsis verbis*; all that is required is that it reasonably convey to persons skilled in the art that, as of the filing date thereof, the inventor had possession of the subject matter later claimed by him." *In re Edwards*, 196 USPQ 465, 467 (C.C.P.A. 1978) (citing *In re Lukach*, 169 USPQ 795 (C.C.P.A. 1971); *In re Driscoll*, 195 USPQ 434 (C.C.P.A. 1977)).

More recently, the Court of Appeals for the Federal Circuit, in *In re Kaslow*, 217 USPQ 1089, 1096 (Fed. Cir. 1983), citing *In re Edwards*, emphasized:

> The test for determining compliance with the written description requirement is whether the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter, rather than the presence or absence of literal support in the specification for the claim language.

More recently, in *In re Alton*, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996), the court of Appeals for the Federal Circuit pointed out that literal support is not required in order to satisfy the written description requirement:

> If a person ordinary skill in the art would have understood the inventor to have been in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate written description requirement is met. For example, in *Ralston Purina Co. v. Far-Mor-Co., Inc.*, 227 USPQ 177, 180 (Fed. Cir. 1985), the trial court admitted expert testimony about known industry standards regarding temperature and pressure in "the art of both farinaceous and proteinaceous vegetable materials." The effect of the testimony was to expand the breadth of the actual written description since it was apparent that the inventor possessed such knowledge of industry standards of temperature and pressure at the time the original application was filed.

More recently, in *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997) cert. denied, 523 U.S. 1089 (1998), the Court of Appeals for the Federal Circuit stated:

> "In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus."

The court reasoned that factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making

the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406; MPEP 2163 II(A)(3)(a)(i).

When species of a genus are claimed, only a representative number of species must be adequately described. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Furthermore, the description of a representative number of species does not require the description to be of such specificity that it need provide individual support for each species of a genus. *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994).

The Examiner has the burden of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. MPEP 2163. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed. In re Wertheim, 541 F.2d 257, 262, 191 USPQ 90, 96.

Therefore, it is clear that the invention need not be described in *ipsis verbis, i.e.*, literally, for purposes of the written description requirement under 35 U.S.C. §112, first paragraph. Rather, what is needed is that the skilled artisan understand, based upon the disclosure in the specification as filed and the knowledge imputed to the skilled artisan at the time the specification was filed, that the inventor had possession of the claimed subject matter.

Applicant respectfully submits that one skilled in the art, upon reading the specification as filed, would have understood that the invention encompassed an endorepellin protein and fragments of endorepellin. As described above, endorepellin protein and endorepellin fragments are supported in the specification as filed. At page 5, the Examiner admits that "Support for fragments, derivatives, and or analogs can be found on page 2 lines 31-33 and page 3 lines 1-3." The specification provides the entire domain V of perlecan as well as seven specific fragments of domain V (endorepellin), including their location within perlecan, in Figures 1f and 1g. Furthermore, as described above, the nucleic acid and amino acid sequences for endorepellin were known in the art at the time the specification was filed. The specification provides relevant molecular and biochemical

methods for preparing endorepellin protein and endorepellin fragments, including specific fragments defined by residue number and by function, as well as multiple assays with which to test the ability of endorepellin protein and endorepellin fragments to inhibit angiogenesis (see pages 3-25). Thus, not only are specific structural elements of endorepellin and endorepellin fragments described, but functional activities are described as well. As discussed above, only a representative number of fragments need be described to provide support for other fragments (*Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406; *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993); *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994)). Because multiple fragments of endorepellin are disclosed in the specification as filed, Applicant asserts that the written description is commensurate in scope to any and all endorepellin fragments.

Regarding the Examiner's assertion that the specification is missing SEQ ID NOS: that specifically identify fragments claimed with an associated sequence, as discussed above, specific sequence information and amino acid residue position information for endorepellin protein and its fragments was provided in the specification as filed, or was known to those of skill in the art at the time the specification was filed. In addition, Applicant respectfully submits that SEQ ID NOS: are not required, and are not needed in the present application. As discussed above, a description need only describe in detail that which is new or not conventional. See *Hybritech v. Monoclonal Antibodies*, 802 F.2d 1367, 1384, 231 USPQ 81, 94; *Fonar Corp. v. General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805. The requirement for the use of sequence listings is merely based on a goal of the Patent Office to build a database of sequences for reference purposes. For example, it is stated in the MPEP that:

> "[t]he goal of the Office is to build a comprehensive database that can be used for, inter alia, the purpose of assessing the prior art. . . . In those instances in which prior art sequences are only referred to in a given application by name and a publication or accession reference, they need not be included as part of the "Sequence Listing, . . ."

MPEP 2422.03, paragraph 6. Because the sequence of endorepellin and it fragments can be found in the prior art (Murdoch et al.), its sequence need not be included as part of this application and the goal of the patent office in assessing prior art has been satisfied.

Furthermore, as detailed above, Figures 1f and 1g of the specification describe specific fragment lengths and positions of the amino acid residues for the amino and carboxy termini of each fragment for endorepellin and fragments of endorepellin disclosed in the invention. For example, the present applications discloses that two of the deletion fragments of endorepellin, Δ1 and Δ5, comprising fragments a.a. 3687-4181 and a.a. 3927-4181, respectively, possess endostatin binding activity (page 19, lines 11-24; Fig. 1f, Fig. 1g.). Also as described above, the nucleic acid and amino acid sequences for perlecan and domain V (endorepellin) of perlecan were known to those of ordinary skill in the art (Murdoch et al.) at the time the specification was filed.

Based upon the disclosure in the specification as filed and the knowledge imputed to the skilled artisan at the time, a skilled artisan would indeed understand that the applicant had possession of the claimed subject matter.

In view of the present specification as filed and the prior art usage as discussed above, one of ordinary skill in the art would readily understand the definition and scope of the claims as filed. Applicant respectfully requests reconsideration and withdrawal of the 35 U.S.C. § 112, first paragraph, written description rejection.

Rejection of Claims 15 and 16 under 35 U.S.C. § 112, first paragraph, enablement

At page 6 of the Office Action, the Examiner has again rejected claim 15, and newly added claim 16, for lack of enablement. Claim 16 has been cancelled, therefore the rejection as to this claim is now moot. The Examiner now asserts that at the time of the invention, several references taught that proteins that have found relative anti-angiogenic success in vitro have had little effect in human trials. The Examiner then alleges that Auerbach et al. states ". . . the interpretation of in vitro success of angiogenic assays, at best, provides an initial assessment of compounds for the ability to function effectively in vivo," citing pages 32-40. The Examiner then states that "Although it is noted that the instant invention provides a semi-in vivo (technically considered an in vitro assay, see Auerbach et al., p 36) analysis of endorepellin in the CAM assay, others have shown that the ability of angiogenic proteins that have potent anti-angiogenic effects in vitro have failed to provide the same response in vivo." The Examiner also refers to Auerbach for the proposition that in vivo analysis is critical for the full interpretation of the effectiveness of a compound. The

Examiner then cites a newspaper article from 1998 as support for his argument regarding proteins regulating angiogenesis.

Applicant respectfully disagrees and submits that claim 15 is enabled by the specification, based on the following reasons.

A specification which discloses how to make and use a claimed invention is presumed to comply with the first paragraph of 35 U.S.C. § 112, unless there is a reason to doubt the objective truth of the specification. *In re Marzocchi*, 439 F.2d 220, 169 USPQ 367 (CCPA 1971). The initial burden of establishing a basis for denying patentability to a claimed invention therefore rests upon the examiner. *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988); *In re Thorpe*, 777 F.2d 695, 227 USPQ 964 (Fed. Cir. 1985); *In re Piasecki*, 745 F.2d 1468, 223 USPQ 785 (Fed. Cir. 1984). Here, the present specification clearly discloses how to make and use the claimed endorepellin peptide and fragments thereof, and how to use them in vitro and in vivo, and the Examiner has failed to rebut the assertions made therein.

It is well-settled that an applicant need not have actually reduced the invention to practice prior to filing in order to satisfy the enablement requirement under 35 U.S.C. §112, first paragraph. MPEP §2164.02 (citing *Gould v. Quigg*, 822 F.2d 1074 (Fed. Cir. 1987)). Indeed, the invention need not contain a single example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation (*In re Borkowski*, 422 F.2d at 908), and "representative samples are not required by the statute and are not an end in themselves" (*In re Robins*, 429 F.2d 452, 456-57, 166 USPQ 552, 555 (CCPA 1970)). Thus, 35 U.S.C. § 112, first paragraph, enablement does not require any working examples.

The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. MPEP §2164.01 (citing *In re Angstadt*, 537 F.2d 498, 504 (C.C.P.A. 1976)). The fact that experimentation may be complex does not necessarily make it undue if the art typically engages in such experimentation. *Id.* Further, the specification need not disclose what is well known to those skilled in the art and preferably omits that which is well-known to those skilled in the art and is already available to the public. MPEP §2164.05(a) (citing *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir.

1991)). Enablement does not require a working example. Experimentation is allowed, so long as it is not undue.

Examiner alleges at pages 6-7 that the specification has failed to demonstrate endorepellin's anti-angiogenic use in vivo. Applicant again asserts that the Examiner is incorrect in this rejection. The CAM assay *is an in vivo assay* which utilizes live chicken embryos and is performed over several days (page 20, lines 18-24; Figure 2d). The CAM assay is an accepted in vivo animal model of angiogenesis and the reasoning is provided below.

First, Applicant respectfully points out that the Examiner's reference to Auerbach's discussion of "in vitro" assay was incomplete and failed to recite the entire statement by Auerbach at p. 36 regarding the CAM assay, in which Auerbach states "Although technically this may be considered an in vitro assay, it is a whole animal assay" (emphasis added). Thus, Examiner has misinterpreted the statement by Auerbach. What Auerbach is stating is that although one might technically call one form of the assay an "in vitro" assay, e.g., the explant method (when the embryo is removed from the egg), the assay is still an in vivo assay because the whole animal is being used. The embryo is merely no longer in the egg.

Furthermore, Applicant also points out that Auerbach does indeed consider the CAM assay as being an in vivo assay. First, Auerbach describes the CAM assay in the section entitled "**In Vivo Assays,**" which starts at page 35 of Auerbach. Auerbach further asserts at p. 36, first column, that the original CAM assay has been a mainstay for in vivo studies for 50 years.

Not only does Auerbach classify the CAM assay as an in vivo assay in the article cited by the Examiner, Auerbach describes the assay as an in vivo assay in other publications as well. For example, see Auerbach et al., Cancer and Metastasis Reviews, 2000, 19:167-172, particularly page 170 (submitted herewith).

The CAM assay is a classical and accepted assay for studying angiogenesis in vivo. In a review entitled "The Chick Embryo Chorioallantoic Membrane as a Model for **In Vivo** Research on Anti-Angiogenesis," it is stated at page 73 that "The classical assays for studying angiogenesis **in vivo** included the hamster cheek pouch, the rabbit ear chamber, dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) and the iris and

avascular cornea of the rodent eye" (Ribatti et al., Current Pharmaceutical Biotechnology, 2000, 1:73-82; emphasis added; submitted herewith).

Furthermore, the CAM assay has been accepted by the USPTO in granting claims to a method of inhibiting angiogenesis in mammals in U.S. Patent No. 6,284,726. The CAM assay was utilized in U.S. Patent No. 6,284,726 (submitted herewith) to demonstrate the anti-angiogenic activity of certain peptide analogs of high molecular weight kininogen domain 5. See column 11, lines 50-51, of U.S. Patent No. 6,284,726: "[t]he effect of the HK domain 5 peptides on cytokine-stimulated angiogenesis in vivo. . ." See Figures 1A to 1D of U.S. Patent No. 6,284,726 for the results of that CAM assay. The undersigned was attorney of record in U.S. Patent No. 6,284,726. Claims directed to pharmaceutical compositions and methods of inhibiting angiogenesis issued on the basis of the data in the specification. The CAM assay was the only in vivo assay utilized in the examples of U.S. Patent No. 6,284,726. No additional data of anti-angiogenic effect was submitted during prosecution.

In addition to the CAM assay being a true in vivo assay demonstrating the effect of endorepellin in the specification as filed, the anti-angiogenic effect of endorepellin peptide has recently been demonstrated in mice using the Matrigel® plug in vivo angiogenesis assay (Mongiat et al., 2003, J. Biol. Chem. 278:6:4238-4239). The Matrigel® plug assay measures growth of the host animal's blood vessels into the plug. In Mongiat et al., Matrigel® plugs containing fibroblast growth factor-2, in the presence or absence of endorepellin, were injected subcutaneously into mice. It was found that endorepellin inhibited neovascularization within and around the Matrigel® plug (Figures 5a to 5d). Thus, the anti-angiogenesis activity of endorepellin has been demonstrated in two different in vivo models.

Furthermore, at the time the specification was filed it was known to those of skill in the art that proteins which inhibit angiogenesis in vitro, in models such as the three-dimensional models described in the specification, would also inhibit angiogenesis in vivo. References cited in the specification demonstrated the effects of angiogenesis-modulating proteins such as perlecan and endostatin with both in vitro and in vivo assays (Aviezer et al., 1994, Cell 79:6:1005-1013; Nugent et al., 2000, Proc. Natl. Acad. Sci. USA 97:6722-6727; O'Reilly et al., 1997, Cell 88:277-285; Yamaguchi et al., 1999, EMBO J. 18:4414-4423).

In sum, Applicant respectfully submits that claim 15 and its new dependent claim are supported by the disclosure provided in the specification as filed. Therefore, undue experimentation would not be required of a skilled artisan to make and/or use the full scope of the invention in vivo as recited in claim 15. Given the advanced state of the relevant art, the ample disclosure, and the extensive reduction to practice provided in the specification as filed, claim 15 is enabled and this requirement of 35 U.S.C. § 112, first paragraph, has been satisfied. Thus, Applicant respectfully requests that the rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Rejection of Claims 15 and 16 under 35 U.S.C. § 102(b), anticipation

Claims 15 and 16 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Murdoch et al. (J. Biol. Chem. 1992 267:12:8544-8557). Claim 16 has been cancelled, therefore the rejection as to this claim is now moot. The examiner acknowledges that the claims are drawn to a pharmaceutical composition comprising endorepellin, also known as domain V of perlecan, wherein the endorepellin protein is between 210-705 amino acids in length. The Examiner alleges that at p. 8550 Murdoch discloses a protein that comprises domain V of perlecan, and also specifically discloses a protein that is 704 amino acids in length. Applicant respectfully disagrees.

For a reference to anticipate a claim, every limitation of that claim must identically appear, either expressly or inherently, in the reference. (MPEP § 2131; In re Bond, 15 USPQ2d 1566, 1567 (Fed. Cir. 1990)). Absence of any claim element from the reference "negates anticipation." Kloster Speedsteel AB v. Crucible, Inc., 230 USPQ 81, 84 (Fed. Cir. 1986); Rowe v. Dror, 42 USPQ2d 1550, 1552 (Fed. Cir. 1992). Here, Murdoch does not disclose every element of claim 15.

Amended claim 15 and its dependent claim are directed to "**a pharmaceutical composition**," comprising an endorepellin protein, wherein the endorepellin protein consists of amino acid residues 3687 to 4391 of the perlecan protein, or an endorepellin fragment, and a pharmaceutically acceptable carrier or excipient. The pharmaceutical composition comprising an endorepellin protein or its fragments is useful for administering endorepellin to a subject.

Applicant respectfully points out that Murdoch merely discloses a protein and a description of the protein. Murdoch does not disclose "a **pharmaceutical composition** comprising an endorepellin . . ." (emphasis added), as is claimed in the present application. Nowhere in Murdoch is a pharmaceutical composition comprising an endorepellin protein or its use disclosed. Nowhere does Murdoch teach or suggest combining a pharmaceutical carrier or excipient with perlecan or a fragment thereof to form a pharmaceutical composition.

Thus, Murdoch does not disclose every element of claim 15. Applicant respectfully submits that claim 15 and its dependent claims are not anticipated by Murdoch under 35 U.S.C. § 102(b) and requests that the rejection be withdrawn.

Claims 15 and 16 also stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Snow (U.S. Patent No. 5,958,883). Claim 16 has been cancelled, therefore the rejection as to this claim is now moot. The Examiner alleges that Snow discloses a perlecan molecule that is administered in vivo. The Examiner reasons that because endorepellin is defined as the carboxy terminus of perlecan or as domain V, and because domain V consists of 704 amino acids, as evidenced by Murdoch, the claims are anticipated by Snow. Applicant respectfully disagrees.

As discussed above, for a reference to anticipate a claim, every limitation of that claim must identically appear, either expressly or inherently, in the reference.

Claim 15 and its dependent claim recite a pharmaceutical composition comprising an endorepellin protein or its fragments.

Snow does not disclose a pharmaceutical composition comprising an endorepellin protein or its fragments, nor does it disclose treating a disease with endorepellin or fragments of endorepellin or treating a disease with a pharmaceutical composition comprising an endorepellin protein or its fragments. Snow is not relevant because it discloses treating various amyloidosis problems with perlecan (for example, see abstract and col. 15, lines 40-50), not with fragments of perlecan such as endorepellin. Thus, Snow does not anticipate claim 15 and its dependent claims.

Claims 15 and 16 also stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Whitelock et al. (WO 99/06054). Claim 16 has been cancelled, therefore the rejection as to this claim is now moot. The Examiner alleges that Whitelock discloses a

pharmaceutical composition comprising perlecan or active fragments thereof in combination with pharmaceutical carriers and diluents. The Examiner further alleges that because endorepellin is also known as domain V of perlecan, Whitelock anticipates the instantly claimed pharmaceutical composition. Applicant respectfully disagrees.

As discussed above, for a reference to anticipate a claim, every limitation of that claim must identically appear, either expressly or inherently, in the reference.

Whitelock discloses perlecan binding to bFGF and the perlecan-bFGF complex stimulating cell proliferation in wound healing (page 3, line 30 to page 4, line 6; page 17, line 25 to page 19, line 8; Figures 5 and 6). Whitelock further discloses that domain I of perlecan (amino terminal domain) is the site of binding of bFGF to perlecan (page 1, lines 12-16; page 3, lines 18-23). Whitelock does not disclose a pharmaceutical composition comprising domain V of perlecan or fragments of domain V of perlecan. In fact, Whitelock only discloses the use of domain I of perlecan, because domain I is required for binding to bFGF and subsequent stimulation of cell proliferation. Thus, the pharmaceutical composition of Whitelock does not contain merely domain V of perlecan, or fragments of domain V of perlecan as claimed in the present application. Whitelock's composition must contain domain I of perlecan.

Furthermore, Whitelock discloses stimulating cell proliferation with a pharmaceutical composition comprising perlecan (page 3, line 30 to page 4, line 6; page 17, line 25 to page 19, line 8; Figures 5 and 6), it does not disclose inhibiting angiogenesis and inhibiting cell proliferation with a pharmaceutical composition comprising domain V of perlecan or fragments of domain V of perlecan as does the present application (Table 1; Figures 2-5; page 19, line 27 to page 21, line 24). Therefore, Whitelock does not anticipate the claims of the present application because it does not disclose a pharmaceutical composition comprising endorepellin or fragments of endorepellin. Furthermore, Whitelock teaches away from the present invention because it focuses on adhesive properties of perlecan and methods to treat wound healing, while the present application discloses the role of endorepellin in inhibiting the adhesion of endothelial cells and subsequently the migration of endothelial cells to form new blood vessels.

Conclusion

Based on the foregoing, all claims under review are believed to be in condition for allowance. An early and favorable action toward that end is earnestly solicited.

Respectfully submitted,

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The Chick Embryo Chorioallantoic Membrane as a Model for *in vivo* Research on Anti-Angiogenesis

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Abstract: Anti-angiogenesis, i.e.inhibition of blood vessel growth, is being investigated as a way to prevent the growth of tumors and other angiogenesis-dependent diseases. Pharmacological inhibition interferes with the angiogenic cascade or the immature neovasculature with synthetic or semi-synthetic substances, endogenous inhibitors or biological antagonists. The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors. Angiogenesis or anti-angiogenesis is evaluated quantitatively or semiquantitatively. The fields of application of CAM in the study of anti-angiogenesis, including our personal experience, are illustrated in this paper.

INTRODUCTION

Angiogenesis is a feature of embryonal development and in several physiological and pathological conditions, including rheumatoid arthritis, psoriasis, tumor growth and metastasis, diabetic retinopathy and age-related macular degeneration [1]. It appears to depend on the balance of several stimulating and inhibiting factors [2]. Angiogenesis-dependent diseases are controlled by using chemotherapy, immunotherapy and radiation therapy to inhibit the stimulating or stimulate the inhibiting factors.

Anti-angiogenesis, i.e inhibition of blood vessel growth, as a way of treating primary tumors and reducing their metastases, was first proposed by Judah Folkman in 1971 [3]. Angiogenesis inhibitors are described as class 1 (specific and semi-specific) and class 2 (non-specific), depending on whether they inhibit proliferation and/or migration of endothelial cells only, or are also toxic for tumor cells [4]. About 20 inhibitors are currently being tested in human trials: most are in early phase I or II clinical studies; some are in or entering phase III testing [5].

The classical assays for studying angiogenesis *in vivo* include the hamster cheek pouch, the rabbit ear chamber, dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) and the iris and avascular cornea of the rodent eye [6]. Several new models have recently been introduced including subcutaneous implantation of various three-dimensional substrates such as polyester sponges, polyvinyl-alcohol foam discs covered on both sides with a Millipore filter (the disc angiogenesis system), and Matrigel, a basement membrane-rich extracellular matrix. The three most widely used assays are CAM, the rabbit corneal micropocket and the subcutaneous implants. The main advantages of the CAM assay are its low cost, simplicity, reliability, lends itself to large-scale screening, which are important determinants of the choise of a method.

CHICK EMBRYO CHORIOALLANTOIC MEMBRANE

The CAM is an extraembryonic membrane formed on day 4 of incubation by fusion of the chorion and the allantois. Since it mediates gas exchanges with the extraembryonic environment until hatching, it has a very thick capillary network that forms a continuous surface in direct contact with the shell. Rapid capillary proliferation continues until day 11; the mitotic index then declines just as rapidly, and the vascular system attains its final arrangement on day 18, just before hatching [7].

IN OVO METHOD

Fertilized White Leghorn chicken eggs are placed in an incubator as soon as embryogenesis starts and are kept under constant humidity at 37°C. On day 3, a square window is opened in the shell after removal of 2-3 ml of albumen to detach

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the CAM from the shell. The window is sealed with a glass and incubation goes on until the day of experiment [8].

IN VITRO UTILIZATION

The embryo and its extraembryonic membranes are transferred to a Petri dish on day 3 or 4 of incubation. CAM develops at the top as a flat membrane and reaches the edge of the dish to provide a two-dimensional monolayer onto which multiple grafts can be placed because the entire membrane can be seen [9].

TESTING SUBSTANCES

The substance is soaked in inert synthetic polymers laid upon the CAM: Elvax 40 (ethylene-vinyl acetate copolymer) and hydron (a poly-2-hydroxyethyl-methacrylate polymer) are commonly used. Both polymers were first described and validated by Langer and Folkman [10]. They are biologically inert and polymerize in the presence of the test substance, allowing its sustained release at constant rates (nanograms to micrograms). When they are combined with an anti-angiogenic substance, the vessels become less dense around the implant and eventually disappear.

Alternatively, a fluid substance can be inoculated directly into the cavity of the allantoic vesicle, so that its activity will develop over the whole vascular area [11]. The anti-angiogenic response affects the CAM vessels as a whole.

In Nguyen *et al.* method [12], a collagen gel is conjugated with the test substance and placed between two parallel nylon meshes. The "sandwich" is then placed upon the CAM on day 8 of incubation. This method quantities the new blood vessels growing vertically into the collagen gel as a percentage of the squares in the top mesh containing a vessel. Since histologic analysis is not required, a large number of compounds can be screened. The effect of an inhibitory substance (placed on the bottom mesh) is quantified by calculating the inhibition of the vasoproliferative response induced by an angiogenic factor, such as fibroblast growth factor-2 (FGF-2). One of the major advantages of the CAM assay is the use of various stimulators alone or in combination with an anti-angiogenic agent to examine the effectiveness of an inhibitor.

We have also described a new quantification method in which gelatin sponges are implanted on top of the growing CAM on day 8 [13]. Blood vessels growing vertically into the sponge and at its boundary with the CAM mesenchyme are counted morphometrically on day 12. The gelatin sponge is also suitable for the delivery of cell suspensions onto the CAM. Furthermore, it is well tolerated and very little, if any, inflammatory reaction occurs. A common problem in the CAM assay is maintenance of the test substance at the site of administration. In the gelatin sponge/CAM assay, it is held within the graft and this adheres firmly to the CAM surface.

The CAM may also be used to verify the ability to inhibit the growth of capillaries by implanting tumors onto the CAM and by comparing tumor growth and vascularization with or without the administration of the anti-angiogenic substance [8].

SEMIQUANTITATIVE EVALUATION OF THE ANTI-ANGIOGENIC RESPONSE

Two independent observers determine the radius of the growth inhibition zone as 0-4 grades of vessel growth from the center of each disk to the furthest contiguous area in which tertiary vessels are absent. Zones with a radius greater than 1 mm are interpreted as evidence of significant inhibition of angiogenesis [14].

QUANTITATIVE EVALUATION OF THE VASOPROLIFERATIVE RESPONSE

Vessel density is quantified by morphometric evaluation of histologic CAM sections fixed at regular intervals after implantation. The total number of vessels in 6 randomly chosen fields are counted. Vessel density is evaluated planimetrically [15] with a 12-line x 12-line reticule inserted in the eyepiece of a photomicroscope. The total number of intersection points in 6 randomly chosen fields occupied by transversally sectioned microvessels 3 to 10 μm in diameter are counted. Vessel number and density are determined by two independent observers and processed statistically.

THE LIMITATIONS OF CAM

The major disadvantage of CAM is that it already contains a well-developed vascular network and the vasodilation that invariably follows its manipulation may be hard to distinguish from the effects of the test substance. Another limitation is nonspecific inflammatory reaction from the implant is that the histologic study of CAM sections demonstrates the presence of perivascular inflammatory infiltrate together

with any hyperplastic reaction of the chorionic epithelium. Nonspecific inflammatory reactions are much less frequent when the implant is made very early in CAM development and the host's immune system is relatively immature [16].

Another drawback is that polymers often do not adhere to the CAM surface. Folkman has suggested to hydrate test substance with 5 µl H_2O on a sterile coverslide glass, which is then turned over and placed on the CAM on day 9-10 [17]. Saline solutions cannot be employed because the hyperosmotic effect of crystal salts damages the chorion epithelium and induces fibroblast proliferation [18]. The substance must thus be used at concentrations of picograms to micrograms, as higher concentrations would cause this hyperosmotic effect [19].

Finally, it might emphasize that species-specific differences might arise if, for example, one attempt to test the effects of high affinity antibodies generated against human surface antigens. However, to circumvent this drawback, it is useful to perform the experiments early in the CAM development, since at that time the host's immune system is relatively immature [16].

TESTING ANTI-ANGIOGENIC SUBSTANCES IN THE CAM ASSAY (TABLE 2)

Angiogenesis is a complex multistep process and as such presents a number of key targets for therapeutic intervention. The broad mechanism, by which anti-angiogenic substances are through to work, are listed in (Table 1).

Table 1. Mechanism of Action of Anti-angiogenic Substances

Interference with angiogenic stimulators
Interference with angiogenic receptors
Interference with the extracellular matrix
Interference with the control of angiogenesis by hypoxic signaling
Interference with proteolysis
Vascular targeting

Table 2. Studies Demonstrating the Anti-angiogenic Activity of various Substances in the CAM Assay

Substance	Reference
VEGF-165 or VEGF-121 DT 385 (Diphteria toxin)	21-22
Anti-FGF-2 antibody	24
Anti-angiogenin antibody	26-30
Anti-PlGF-1 antibody	32
Interleukin-2	33
Angiostatin	36
Endostatin	37
Steroids and heparin	39-42
Heparan sulfate	43
Protamine sulfate	44-45
Platelet factor-4	46
Heparanase inhibitors	48
Pentosan poly sulfate	49
GM 1474	50
Non- or low-sulfated saccharides	51
Inhibitor of arylsulfatase	55
Sulfated polysaccharide-peptidoglycan	56
Alpha, beta, gamma-cyclodextrin	57
Suramin	58-62
Spironolactone	63
Tyrosine kinase inhibitors	64-67
Antagonists of adhesion molecules	68-72
Matrix metalloproteinase inhibitors	73-74

(Table 2). contd....

Substance	Reference
Somatostatin	75-78
Nitric oxide	79-81
Anticancer agents	82-88
Hormones	89-90
Antibiotics	91-95
Cartilage	96-101
Thalidomide	102
Cyclosporin	103

To assess anti-angiogenic effects, noninvasive methods, including quantitation of angiogenic growth factors in serum and urine may be also used.

The more promising anti-angiogenic substances belong to the category of naturally occurring inhibitors include angiostatin and endostatin. They are highly specific for activated endothelial cells, have low toxicity and do not cause immunological response.

Antibodies to Angiogenic Stimulators

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a heparin-binding angiogenic factor with endothelial target specificity [20]. The **VEGF-165 or VEGF-121 DT 385 (Diphteria toxin)** conjugate blocks FGF-2-induced angiogenesis in the CAM [21-22].

Fibroblast growth factors (FGFs) are a family of heparin-binding polypeptides. **FGF-2** exerts angiogenic activity *in vivo* and induces cell proliferation, protease production and chemotaxis in endothelial cells *in vitro* [23]. A rabbit polyclonal **anti-FGF-2 antibody** inhibits angiogenesis in the CAM [24].

Angiogenin is a polypeptide isolated for the first time from the culture medium of a human adenocarcinoma cell line [25]. A **monoclonal antibody** to human **angiogenin**, synthetic peptides corresponding to the C-terminal region of angiogenin and a peptide complementary to its receptor-binding site inhibits angiogenin-induced neovascularization in the CAM. Replacement of His-13 and His-114 in the ribonucleolytic and angiogenic activities of angiogenin and human placental ribonuclease inhibitor abolishes angiogenic activity in the CAM [26-30].

Placental-derived growth factor (PlGF) is a dymeric angiogenic heparin-binding glycoprotein showing a high degree of sequence similarity to the VEGF (31). An affinity-purified **anti-PlGF-1 antibody** inhibits angiogenesis in the CAM [32].

Naturally Occurring Inhibitors of Angiogenesis

Interleukin-2 (IL-2) has a slight effect on angiogenesis *in vivo* in the rabbit cornea model [33]. IL-2 inhibits angiogenesis in the CAM in a dose-dependent manner [34].

Angiostatin, a specific inhibitor of endothelial cell proliferation, is an internal fragment of mouse plasminogen, comprising the first four disulfide-linked kringle domain [35]. It inhibits angiogenesis in a number of primary and metastatic tumors [36].

Endostatin is a C-terminal fragment of collagen XVIII; it specifically inhibits endothelial cell proliferation and is a potent inhibitor of angiogenesis and tumor growth [37].

Angiostatin and endostatin have been demonstrated to induce tumor regression and tumor dormancy without drug resistance in several experimental models. Both inhibit angiogenesis in the CAM [36-37].

Synthetic and Small Molecular Weight Inhibitors

Sulfated analogs. A wide range of cellular functions including growth, morphology and migration are modulated by **heparin (HE)** and **heparan sulfate** [38]. HE consists of a mixture of polysulfated 6 to 20 kDa polysaccharides. Variations in the size of the polysaccharide chain and in the degree and distribution of sulfate groups contribute to a high degree of heterogeneity. HE alone may stimulate, inhibit or have no effect on angiogenesis *in vivo*. It binds angiogenic growth factors, including FGFs, VEGF, hepatocyte growth factor/scatter factor (HGF/SF) and the human immunodeficiency virus-1 transactivating factor tat. HE fractioned into low and high molecular weight species may inhibit or facilitate the binding of HE-binding growth factors to their receptors. Low molecular weight HE, for example, suppresses FGF-2-mediated angiogenesis more effectively. HE affects endothelial cell

proliferation and motility *in vitro* and modulates neovascularization *in vivo* when administered with certain **corticosteroids**.

CAMs treated with combinations of **angiostatic steroids** and **HE** reduce their vascularity and exhibit capillary **basement membrane fragmentation** and complete loss of fibronectin and laminin from the region of capillary involution. HE plus cortisone acetate and cortisone plus **hexasaccharide** inhibit angiogenesis, whereas HE, cortisone or hexasaccharide alone are non-anti-angiogenic [39-41]. **HE plus cortisone** induces a marked depression in the rate of collagenous protein biosynthesis in the CAM [42]. **HE** has an anti-angiogenic effect by itself, and an additive effect is obtained when it is combined with hydrocortisone. **Heparan sulfate** also has an anti-angiogenic effect, whereas **keratan sulfate, dermatan sulfate or chondroitin sulfate** have none [43].

Protamine and **platelet factor-4**, proteins that bind avidily to HE, inhibit angiogenesis. **Protamine sulfate** inhibits angiogenesis in the CAM [44-45]. Recombinant human **platelet factor-4** (rPF-4) inhibits angiogenesis in the CAM in a dose-dependent manner [46]. Both rPF-4 and an analog lacking affinity for HE (rPF4-241) inhibit angiogenesis in the CAM. The analog is inhibitory at lower concentrations than rPF4 and its inhibitory effects are not abrogated by the presence of HE [47].

Some of the most recent modifications of HE have focused on enhancing **heparanase** inhibitory activity. **Heparanase inhibitors** are anti-angiogenic in the CAM [48].

Pentosan polysulfate (PPS) is a **HE analog** used preclinically as an anticoagulant. It inhibits angiogenesis in the CAM [49].

GM 1474 is a low molecular weight **polysulfated oligosaccharide** that also binds to FGF-2. It inhibits angiogenesis in the CAM [50].

The anti-angiogenic effect of **non- or low-sulfated saccharides** is unaffected by the addition of **hydrocortisone**. **K5 polysaccharide**, its fragments down to octasaccharide size, and analogous N-acetylated fragments of heparan sulfate all show anti-angiogenic activity in the CAM. Hyaluronan, however, with the isomeric -(GlcA beta-1,3 GlcNA beta 1,4) (n) was inactive. The anti-angiogenic activity of -(GlcA beta-1,4 GlcNAc delta 1,4)-containing saccharides is potentiated by the presence of L-iduronic acid and one or two o-sulfate groups in the non-reducing terminal disaccharide unit [51].

The **heparan sulfate sulfheparoid** inhibits angiogenesis in the CAM [52]. A **sulfated polysaccharide-peptidoglycan complex (PS-4152)**, in the presence of **cortisone** or **tetrahydro S**, inhibits angiogenesis in the CAM [53-54].

A synthetic **inhibitor of arylsulfatase** (HNT), potentiates the anti-angiogenic activity of a mixture of **heparin** and **hydrocortisone** applied to the CAM in a dose-dependent manner. Hydrocortisone and HNT inhibit angiogenesis to the same extent as hydrocortisone and heparin. Preincubation of heparin with arylsulfatase causes 50% reduction in anti-angiogenic activity of heparin-steroid mixture applied to the CAM. This loss of activity is completely prevented by addition of HNT [55].

Angiogenesis induced by **Kaposi's sarcoma-derived spindle-shaped cells** in the CAM is blocked by a **sulfated polysaccharide-peptidoglycan** compound produced by bacteria [56].

Alpha, beta and gamma-cyclodextrin derivatives have been examined for their angiostatic activity in combination with **hydrocortisone** in the CAM [57].

The most thoroughly studied small-molecule sulfate inhibitor of angiogenesis is **suramin**, a polysulfonated naphthylurea used in the treatment of trypanosomiasis. Suramin alone shows anti-angiogenic activity in the CAM in a dose-dependent manner. It also potentiates the activity of the angiostatic steroids, hydrocortisone, cortisol-21-phosphate, 17-alpha-hydroxyprogesterone, tetrahydrocortisol, and tetrahydrocortexolone. HE decreases its angiostatic activity. **Eriochrome black T** (EBT), structurally related to suramin and suramin analogs, are more potent and less toxic than suramin in the CAM [58-62].

Steroids, flavinoids and steroid conjugates. Steroids are among the first small-molecules that show an anti-angiogenic effect *in vivo*. **Spironolactone** is an orally active, renal aldosterone antagonist used to reat hypertension, congestive heart failure and other diseases. It inhibits angiogenesis in the CAM [63].

Tyrosine kinase inhibitors. Protein tyrosine kinases are involved in induction of angiogenesis. **Staurosporine** and **erbastatin** inhibit angiogenesis in the CAM [64-65]. A series of compounds, originally studied as potential **PKC inhibitors**, including **diaminoanthraquinone NSC 639666**, inhibit angiogenesis in the CAM [66]. **PD98059**, a MEK inhibitor, inhibits FGF-2-induced angiogenesis in the CAM [67].

Adhesion molecules. The role of adhesion molecules (**selectins, immunoglobulin supergene family, cadherins** and **integrins**) in angiogenesis has been established. Analogs of the selectin ligand Sialyl Lewis X inhibited angiogenesis in the CAM [68]. **Integrin** $\alpha_v\beta_3$ allows endothelial cells to interact with a wide variety of extracellular matrix components. Endothelial cells exposed to growth factors or those undergoing angiogenesis express high levels of $\alpha_v\beta_3$. **Cyclic peptide** or **monoclonal antibody (LM 609)** against $\alpha_v\beta_3$ inhibits basal and TNF-α induced angiogenesis in the CAM. **Triflavin**, a member of the disintegrin family, inhibits TNF-α induced angiogenesis in the CAM. **Nonpeptide integrin antagonists** inhibit angiogenesis in the CAM [69-72].

Matrix metalloproteinase (MMP) inhibitors. **MMPs** are a series of zinc-requiring proteolytic enzymes, that are secreted in latent pro-enzyme form and are involved in remodeling and degradation of extracellular matrices. To the extent that proteolysis is an important component of angiogenesis, it can be argued that inhibitors of proteolytic activity should inhibit neovascularization. **Tissue inhibitor of MMP-3** inhibits FGF-2 induced angiogenesis in the CAM assay [73]. A **fragment of MMP-2 (PEX)**, a non-catalytic C-terminal hemopexin-like domain of MMP-2 with integrin binding activity, inhibits MMP-2 activity in the CAM, where it inhibits angiogenesis and tumor growth [74].

Miscellaneous Agents

Somatostatin and its analogs seem to be active in the inhibition of certain tumors. **Somatostatin analogs** SM 201-995, RC-160 and **octreotide acetate** inhibit angiogenesis in the CAM in a dose-dependent manner and show statistically significant inhibition of neovascularization when compared to native somatostatin 14. Furthermore, octreotide inhibits CAM neovascularization by human MCF-10A (int-2) mammary cells secreting FGF-3 [75-78].

Nitric oxide (NO) is an endogenous mediator released from a variety of cell types including endothelial cells, smooth muscle cells, platelets, macrophages and nerve cells of the peripheral and central nervous system. The nitrovasodilators **sodium nitroprusside** (NaNP), **isosorbide mononitrate** (ISMN) and **dinitrate** (ISDN), which release NO spontaneously, and the amino-acid **L-arginine**, inhibit angiogenesis in the CAM. Furthermore, NaNP, ISMN and ISDN completely reverse the angiogenic effect of alpha-thrombin and the protein kinase C (PKC) activator 4-beta-phorbol-12-myristate-13 acetate [79-81].

Anticancer agents. Most anticancer agents are screened for their antiproliferative and differentiation-inducing activity on tumor cells, but not for their differential effects on vascular endothelium. Several cytostatic agents such as **doxorubicin (daunorubicin, epirubicin), mitoxantrone, etoposide, vincristine and vinblastine** are angiostatic in the CAM [82-83].

Immunoconjugate of doxorubicin on the galactose residues of a monoclonal antibody, specific for the tumor-associated carcino-embryonic antigen induces a reduction of tumor-induced angiogenesis and tumor progression in the CAM [84]. Antitumor agent **titanocene dichloride** [85-86], **taxol** [87] and the antineoplastic ether lipid **S-phosphonate** [88] inhibit angiogenesis in the CAM.

Hormones. The non-steroidal antiestrogens, especially tamoxifen, have been extensively used in breast cancer therapy, since they compete with endogenous estrogens for the estrogen receptor. Many recent studies have shown that antiestrogens affect the activity of many growth factors of importance in the control of cell proliferation. Partial **estrogen antagonists, clomiphene, tamoxifen, nafoxidine** and the pure estrogen antagonists, **ICI 164, 384 and ICI 182,780**, inhibit angiogenesis in the CAM in a dose-dependent manner [89]. **2-methoxyestradiol**, an endogenous metabolite of estradiol-17 beta, inhibits FGF-2 induced angiogenesis in the CAM [90].

Antibiotics. Some antibiotics have anti-angiogenic properties. **TNP-470 (AGM-1470)**, a synthetic analog of fumagillin isolated from *Aspergillus fumigatus*, is a potent angiogenesis inhibitor *in vitro* and acts by preventing the entry of endothelial cells into the G1 phase. Locally administered TNP-470 [91] and medium-chain triglyceride (MTC) of TNP-470 [92] inhibit angiogenesis in the CAM. **FR-111142**, a new angiogenesis inhibitor [93] produced by the fungus *Scolecobasidium Arenarlum* F-2015, **neomycin**, an **aminoglycoside antibiotic** [94], and **depudecin, a microbial metabolite** [95], inhibit angiogenesis in the CAM.

Cartilage. Cartilage implants inhibit basal angiogenesis in the CAM and angiogenesis induced by implants of Walker carcinoma or tumor angiogenesis factor (TAF). A factor in conditioned medium of rabbit costal **chondrocytes** inhibits angiogenesis induced in the CAM by B16 melanoma and by tumor transplants [96-97]. An angiogenesis inhibitor, isolated from the conditioned media of scapular chondrocytes, is angiostatic in the CAM [98]. Conditioned medium from a clonal **human chondrosarcoma cell line**, inhibits angiogenesis induced in the CAM by B16 melanoma [99]. A potent angiogenesis inhibitor,

U995, purified from the cartilage of the blue shark, inhibits TNF-α induces angiogenesis in the CAM [100]. Purified recombinant human **chondromodulin-1** (ChM-1), purified from fetal cartilage, inhibits angiogenesis in the CAM [101].

Thalidomide, a well-known, potent teratogen inhibits angiogenesis in the CAM [102].

Cyclosporin is mainly known as immunosuppressive agent and is widely used in organ transplantation. It inhibits angiogenesis in the CAM [103].

CONCLUDING REMARKS

CAM is widely utilized as an *in vivo* system to study anti-angiogenesis. The rabbit cornea pocket assay [6] is used just as often as an *in vivo* system. CAM, however, offers the advantage of being relatively inexpensive and lends itself to large-scale screening, while the very few restrictions to its use are essentially due to nonspecific inflammatory reactions and to the presence of pre-existing vessels which make it difficult to determine the true extent of anti-angiogenesis.

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Endorepellin, a Novel Inhibitor of Angiogenesis Derived from the C Terminus of Perlecan*

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Perlecan, a ubiquitous basement membrane heparan sulfate proteoglycan, plays key roles in blood vessel growth and structural integrity. We discovered that the C terminus of perlecan potently inhibited four aspects of angiogenesis: endothelial cell migration, collagen-induced endothelial tube morphogenesis, and blood vessel growth in the chorioallantoic membrane and in Matrigel plug assays. The C terminus of perlecan was active at nanomolar concentrations and blocked endothelial cell adhesion to fibronectin and type I collagen, without directly binding to either protein; henceforth we have named it "endorepellin." We also found that endothelial cells possess a significant number of high affinity (K_d of 11 nM) binding sites for endorepellin and that endorepellin binds endostatin and counteracts its anti-angiogenic effects. Thus, endorepellin represents a novel anti-angiogenic product, which may retard tumor neovascularization and hence tumor growth *in vivo*.

Perlecan is a modular proteoglycan that participates in the formation and maintenance of basement membranes in various organs (1–5). The protein modules of perlecan have striking homology to polypeptides involved in lipid uptake, growth control, cell-cell interactions, and adhesion (6–8). Its highly refined molecular architecture, coupled with its ubiquity, suggests that perlecan may play key biological functions during ontogeny, tissue remodeling or transformation (9, 10). Lack of perlecan causes embryonic lethality with severe cephalic and cartilage abnormalities (11, 12). Although basement membranes can develop in the absence of perlecan, the majority of the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and intraventricular pressure rises (12). A recent report (13) has shown that perlecan-null animals exhibit a high incidence of malformations of the cardiac outflow tract with complete transposition of great vessels in ~73% of the embryos, further stressing the key role of perlecan in vasculogenesis. In adult tissues, perlecan is a major heparan sulfate proteoglycan secreted by endothelial cells and is a potent inhibitor of smooth muscle cell proliferation, a biological function mediated by perlecan's block of FGF2[1] activity (14) and *Oct-1* gene expression (15). Indeed, perlecan is a major candidate for the FGF2 low affinity receptor and can restore high affinity binding of FGF2 to its receptor in heparan sulfate-deficient cells (16). FGF2 binds to the heparan sulfate chains attached to the N-terminal domain I, and the bioavailability of this powerful angiogenic factor is modulated by the concerted action of heparanases and proteases (17). In agreement with these data, perlecan plays a critical role in regulating the vascular response to injury *in vivo* (18).

Perlecan is highly enriched in various tumorigenic cell lines (19, 20) and human tumors (21, 22), and blocking the endogenous production of perlecan suppresses autocrine and paracrine functions of FGF2 and impairs tumor cell growth and invasion (23, 24). Likewise, antisense targeting of the perlecan gene causes a marked attenuation of colon carcinoma cell growth, and these effects correlate with a reduced mitogenic response to FGF7 (25). Perlecan protein core binds to FGF7 (K_d ~60 nM) (26), and is required for functional activation of the FGF7 receptor and downstream signaling (27). In addition, the perlecan protein core binds several extracellular matrix molecules and growth factors (28) and acts as either an adhesive or counter-adhesive molecule (29–32).

Angiogenesis is one of the most important events in tumor progression and is greatly influenced by cell matrix interactions taking place at the surface of the endothelial cells and the tumor-matrix boundaries (33). Heparan sulfate proteoglycans act as depots for pro- and anti-angiogenic factors (34, 35) and, in concert with members of the FGF and VEGF family and their receptors, modulate various steps of angiogenesis (10). Because expression of the full-length perlecan is very difficult since its mRNA is over 15 kb, we utilized its C-terminal domain V to search for physiologically relevant binding partners. Domain V is the major cell-binding domain and is potentially a very interactive molecule since it is organized in a structure similar to that of agrin and various types of laminin, and it also binds heparin and α-dystroglycan among other molecules (36, 37). Thus, we employed this fragment to screen a cDNA library in order to identify other putative interacting proteins and to further investigate the function and importance of this protein in the cross-talk between extracellular matrix proteins. Using

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[1] The abbreviations used are: FGF2, fibroblast growth factor 2; LG, laminin-G like module; CAM, chorioallantoic membrane; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; BSA, bovine serum albumin; AP, alkaline phosphatase; HA, hemagglutinin; NTA, nitrilotriacetic acid; ELISA, enzyme-linked immunosorbent assay; BS^3, bis-sulfosuccimidyl-suberate.

the yeast two-hybrid system, we identified collagen XVIII, including the anti-angiogenic factor endostatin, as a strong candidate. We discovered that the C terminus of perlecan, henceforth named "endorepellin," counteracted the anti-angiogenic effects of endostatin, while by itself potently inhibited four aspects of angiogenesis: endothelial cell migration, collagen-induced endothelial tube morphogenesis, and blood vessel growth in the chorioallantoic membrane and in Matrigel plug assays. Endorepellin inhibited angiogenesis at nanomolar concentrations and interfered with endothelial cell adhesive properties for various substrata, including fibronectin and type I collagen. Moreover, endothelial cells possess a significant number of high affinity (K_d of 11 nM) binding sites for endorepellin, which could be cross-linked with bis-sulfosuccimidyl-suberate (BS^3) to form high M_r complexes. Thus, endorepellin may represent a novel anti-angiogenic tool against cancer.

EXPERIMENTAL PROCEDURES

Cells, Yeast Two-hybrid Screening, and Co-immunoprecipitation—Primary cultures of HUVECs were prepared from fresh umbilical cords and cultured on gelatin-coated flasks in M199 or M200 media (Invitrogen) supplemented with 10% fetal bovine serum, 50 μg/ml heparin, and endothelial cell growth supplement, isolated from bovine hypothalami. Only passages 4–8 were used. A431 squamous carcinoma, HeLa squamous carcinoma, HT1080 fibrosarcoma, WiDr colon carcinoma, MCF7 breast carcinoma, and M2 mouse melanoma cells were obtained from American Type Culture Collection (Manassas, VA). We employed the Matchmaker GAL4 two-hybrid system 3 (Clontech, Palo Alto, CA), which adopts three independent reporter genes (His, Ade, and either α- or β-galactosidase) for selection. Endorepellin, subcloned into the pGBKT7 vector, was used as bait to screen ~5 × 10^6 cDNAs from a keratinocyte library constructed in the pACT2 vector. The clones growing in selective medium were replated in quadruple minus plates containing X-α-gal. Interacting cDNAs were identified by automatic sequencing. Deletion mutants were generated by PCR using oligonucleotides, which included suitable restriction sites to allow unidirectional ligation into the pGBKT7 vector (38). Various constructs were *in vitro* transcribed and translated in the presence of [^{35}S]methionine (ICN Pharmaceuticals, Costa Mesa, CA) employing the TnT® reticulocyte lysate system (Promega, Madison, WI). Aliquots were co-precipitated with affinity-purified, anti-hemagglutinin (αHA) rabbit polyclonal antibodies (Clontech). The immune complexes were captured with protein A/G-agarose beads (Pierce), washed with 10 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 200 mM Na_3VO_4, 20 mM NaF, and a protease inhibitor mixture (Roche Diagnostics GmbH, Mannheim, Germany), and separated in polyacrylamide gels. The gels were fixed in ethanol/acidic acid, incubated for 20 min with AMPLIFY™ (Amersham Biosciences), dried under vacuum, and exposed to Kodak films.

Expression and Purification of Recombinant Proteins—The pCEP-Pu vector bearing the sequence of the BM40 signal peptide and the full-length domain V/endorepellin was electroporated into 0.5 ~10^6 human embryonic kidney cells (293-EBNA) expressing the Epstein-Barr virus nuclear antigen (EBNA)-1. The endostatin fragment was cloned by PCR using clone A3 as a template. The following oligonucleotides were used: forward, 5′-CTAGCTAGCCCACAGCCACCGCGACTT-3′; containing an *Nhe*I site and reverse, 5′-CCGCTCGAGTACTTGGAGGCAGTCATGA-3′ containing an *Xho*I site. The GC-Rich PCR system was used (Roche Diagnostics). Mass cultures were selected in media containing 250 μg ml^{-1} G418 and 500 ng ml^{-1} puromycin. Serum-free conditioned media were concentrated in a dialysis bag with polyethylene glycol, dialyzed, and purified on Ni-NTA resin eluted with 250 mM imidazole. In all the purification steps we used phenylmethylsulfonyl fluoride (2 mM) and *N*-ethylmaleimide (2 mM) as protease inhibitors. Using this procedure, we routinely purified 5–10 mg of endorepellin or endostatin $liter^{-1}$ of conditioned medium. ELISA and immunoblotting with anti-domain V (17) or penta-His (Qiagen, Valenica, CA) monoclonal antibodies were performed as described before (26). About 15 pmol of purified LG3 fragment, following separation in 10% SDS-PAGE and electroblotting onto a Problott polyvinylidene difluoride membrane, were microsequenced using Edman degradation (Applied Biosystems, Model 477A) at the protein chemistry facility of the Kimmel Cancer Center. Purified perlecan domain V (~10 μg) was diluted in 50 mM Tris-HCl, containing 150 mM NaCl and further purified through a DEAE Sephacel pre-equilibrated in the same buffer. Following an extensive wash, the material was eluted with 2 M NaCl. Aliquots of each fraction were analyzed by SDS-PAGE.

Endothelial Cell Migration, Tube Formation, Chorioallantoic Membrane (CAM), and Matrigel Plug Assays—A 48-well Boyden chamber (Neuroprobe Inc., Gaithersburg, MD) was used for HUVEC migration assays with $VEGF_{165}$ (R&D Systems, Minneapolis, MN) as a chemoattractant. HUVECs migrated through 8-μm nucleopore, polyvinylpyrrolidine-free polycarbonate filters (Corning, Cambridge, MA) precoated for 48 h with 100 μg ml^{-1} collagen type I (Collaborative Biomedical Products, Bedford, MA). About 2 × 10^4 HUVECs were preincubated for 1 h with different concentrations of endorepellin and/or endostatin from *Pichia pastoris* (Calbiochem-Novabiochem, San Diego, CA), and allowed to migrate through the filter for 6 h at 37 °C in 5% CO_2 with or without $VEGF_{165}$ (10 ng ml^{-1}) in the lower chambers. The filters were then washed, fixed, stained with Diff-Quick stain (VWR Scientific Products, Bridgeport, NJ), and the transmigrated cells were counted using conventional microscopy. For *in vitro* tube-like formation, ~4 × 10^5 HUVECs were seeded for 18 h onto 12-well dishes coated with 100 μg ml^{-1} collagen type I and then covered with a second layer of collagen gel (39). Cultures were incubated until gels had solidified, typically 15–30 min, and then given 1 ml of serum-free media with the various test agents and control substances. For the CAM assays, fertilized White Leghorn chick eggs were incubated at 37 °C. After 3 days of incubation, ~3 ml of albumin were removed to detach the CAM, and a small square window was formed. The window was then sealed with tape, and the eggs were returned to the incubator. At day 9, a 1-mm^3 Gelfoam sterile sponge (Gelfoam, Upjohn Company, Kalamazoo, MI) was placed on the CAMs, and various test factors were applied. In addition, CAM assays were performed using sponges containing either 0.5 × 10^6 WiDr colon carcinoma cells alone or in combination with 3 μg of recombinant endorepellin. Three-day-old embryos (n = 20) were used in each experiment. The mean vessel area was calculated using the NIH Image software program (version 1.61) using at least four embryos per experimental point. Ten squares of ~500 μm^2 each were randomly selected around the sponge area and digitized. The background was uniformly adjusted so that it would appear white, whereas the vessels would be black. The pixel density of the vascularized areas was measured, and the values were finally converted into surface area (μm^2). Student's two-sided t test was used to compare the values of the experimental and control samples. A value of $p < 0.05$ was considered as statistically significant. Matrigel plug assays were essentially performed as previously described (40). Briefly, 100 μl of Matrigel (BD PharMingen, San Diego, CA) containing FGF2 (10 ng/animal), in the presence or absence of endorepellin (12 μg/animal), were injected into the dorsal subcutaneous regions of ten nu/nu mice. Mice were sacrificed 2 weeks after the injection, and the skin was removed to analyze the blood vessel formation. The skin samples were photographed, fixed in buffered formaldehyde, and processed for light microscopy. The newly formed blood vessels present in the Matrigel plug were counted as detailed above.

Binding Studies, Covalent Affinity Cross-linking, and Cell Adhesion Assays—Endorepellin (10 μg) was labeled to high specific activity (~10^{18} cpm mol^{-1}) using Iodogen-coated tubes (Pierce). For saturation binding and Scatchard analysis, confluent HUVECs in 24-well plates were incubated with increasing concentrations of ^{125}I-endorepellin for 2.5 h at 4 °C in M199 containing 0.1% BSA, washed several times, and extracted in the presence of protease inhibitors (41). Estimates of receptor affinity and total binding capacity were made with Sigma Plot 5.0 software. For covalent affinity cross-linking, HUVECs were incubated with various concentrations of ^{125}I-endorepellin for 2 h, and then incubated for 30 min at 4 °C with 20 mM BS^3, a membrane-impermeable cross-linker. After termination of the reaction with 1 M Tris, pH 7.5, the cells were solubilized as described above, and the cross-linked material was separated on SDS-PAGE and visualized by autoradiography (27). Displacement of HUVEC-bound ^{125}I-endorepellin was performed by incubating confluent HUVECs (~10^5 cells per dish) with ^{125}I-endorepellin (5 nM) plus increasing concentrations of recombinant unlabeled endorepellin. The cells were incubated at 4 °C for 3 h, washed three times, extracted, and counted in total as above. HUVECs and various tumor cell lines were tested for adhesion to various substrata using various coating concentrations (10–180 nM) of fibronectin, collagen type I, BSA, endorepellin or endostatin as plastic-immobilized substrata. The adhesion assays were conducted in serum-free M199 medium. About 5 × 10^4 cells were plated in quadruplicate wells and, after 1 h of incubation, adherent cells were washed, fixed in 1% glutaraldehyde for 10 min, stained with crystal violet, lysed with 0.2% Triton X-100, and assayed by a colorimetric test (36). The anti-adhesive assays were performed in a similar way on fibronectin-coated plates. After blockage with 1% BSA, the cells were added to the wells in the presence of

increasing concentration of endorepellin or endostatin. After 1 h of incubation, the wells were treated as above.

Expression of Endorepellin/Alkaline Phosphatase Chimeric Protein and Binding Studies—The heat-stable human placental alkaline phosphatase (AP) was amplified by PCR from our previously described construct (42) and ligated in-frame onto the C terminus of endorepellin in the pCEP-Pu vector. The construct was electroporated into 293-EBNA cells as described above. Following several weeks in selective medium (250 μg ml^{-1} G418 and 500 ng ml^{-1} puromycin), several endorepellin/AP-expressing clones were identified using the Great EscAPe™ SEAP system (Clontech), which detects AP. Briefly, conditioned media from untransfected cells (negative control) and from stably transfected cells secreting either AP (positive control) or endorepellin/AP chimera were incubated at 65 °C for 30 min to inactivate endogenous phosphatases, cooled on ice, and then mixed with CSPD substrate/chemiluminescent enhancer for 10 min, followed by exposure to x-ray film for 5–10 s. In addition, the nature of the AP alone and the chimeric protein was identified following immunoprecipitation with a mouse monoclonal antibody (Clone 8B6, Sigma) against human placental AP linked to agarose beads. Binding studies were performed using various cell lines incubated with 0.5 ml of serum-free media conditioned by expressing or control 293-EBNA cells for 48 h. After a 1.5-h incubation at 25 °C, the cells were washed six times, lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, and processed as stated above.

RESULTS

Endostatin Is a Novel Interacting Partner for Perlecan Domain V/Endorepellin—To discover novel interacting partners for perlecan protein core we utilized the entire domain V of perlecan (7, 8), which we named endorepellin (amino acids 3687–4391, Fig. 1*a*) as bait and screened a keratinocyte cDNA library in the yeast two-hybrid system. This domain consists of three laminin-type G (LG1-LG3) modules separated by four EGF-like (EG1-EG4) modules, in an arrangement highly conserved across species (2, 43). One of the strongest interacting clones (clone A3) encoded the C-terminal half of collagen type XVIII, including the NC1 domain containing the potent anti-angiogenic factor endostatin (Fig. 1*b*). Because endostatin inhibits endothelial cell proliferation and effectively arrests the growth of several tumors (44), and because perlecan and endostatin co-localize in most basement membranes (2, 3, 45, 46), we reasoned that an interaction between these two proteins could occur *in vivo* and could play a role in tumor progression. Therefore, we subcloned the collagen fragment into the pGADT7 vector, and the interaction with endorepellin was once more tested with the yeast two-hybrid system on a one-to-one basis. The growth of the cells in quadruple minus medium was comparable to that of the positive control (pGBKT7–53/pGADT7-T), as well as the blue color generated by α-galactosidase expression (Fig. 1*c*). To corroborate the yeast interaction, we *in vitro* transcribed and translated endorepellin and collagen XVIII (clone A3), showing the ~81- and ~65-kDa fragments, respectively (Fig. 1*d*). We could co-precipitate the two proteins with an anti-HA antibody that recognizes the oligopeptide epitope HA present at the C terminus of collagen XVIII (Fig. 1*d*). To determine whether endostatin, which is encoded by the C terminus of collagen type XVIII (Fig. 1*b*), could interact with endorepellin, we cloned the endostatin sequence into pGADT7 vector, and then *in vitro* transcribed and translated the insert, which generated a 23-kDa band (Fig. 1*e*, *lane 3*). As a further control, we subcloned domain III of perlecan into the pGBKT7 vector and then *in vitro* transcribed and translated the insert, which gave the expected ~130-kDa peptide (Fig. 1*e*, *lane 1*). The results showed that only endorepellin interacted with endostatin (Fig. 1*e*, *lane 5*). In contrast, domain III of human perlecan did not bind (*lane 4*).

To establish a direct interaction between endorepellin and endostatin, we performed several solid-phase binding assays using ^{125}I-endorepellin as the soluble ligand, and endostatin, fibronectin or collagen I as the solid substrates coated onto Immulon wells. In these experiments, recombinant endorepellin and endostatin were generated in 293-EBNA cells (see below), and the former was radioiodinated to reach specific activities of ~10^{18} cpm mol^{-1}. We found a saturable binding of ^{125}I-endorepellin to endostatin in the 60–70 nM range, with half maximal binding of ~48 nm (Fig. 1*f*). Specificity of binding was determined by competition experiments with 25-molar excess of cold endorepellin (Fig. 1*f*). In contrast, endorepellin did not substantially bind to either fibronectin or collagen type I (Fig. 1*g*).

Endostatin Interacts Specifically with the LG2 Module of Endorepellin—To establish the precise location of this interaction, we generated seven deletion mutants (Δ1–Δ7) of domain V/endorepellin (Fig. 2). Robust growth in quadruple minus media was observed in cells co-transformed with full-length endorepellin and Δ1 and Δ5, the only two mutants that encompassed the LG2 module (Fig. 2*a*). These results were corroborated by α- and β-galactosidase assays (Fig. 2*b*). In addition to growth in amino acid-deficient media, transcription of *LacZ* (α- and β-galactosidase) under the control of distinct *GAL4* upstream-activating sequences, and the subsequent ability of the co-transformant yeast strains to express functional galactosidase activity, provides an additional strong proof of a true protein-protein interaction (47). Thus, the LG2 module of endorepellin is likely to be the specific site of endostatin binding, although in the native perlecan core protein, the role of the flanking sequences remains to be established.

Recombinant Endorepellin Is Anti-angiogenic—Human recombinant endorepellin, generated in 293-EBNA cells, migrated on SDS-PAGE as a single band of the predicted ~81 kDa, whose identity was further confirmed by immunoblotting with anti-His$_6$ antibody (Fig. 3*a*) and ELISA using a specific monoclonal antibody against domain V (17) (not shown). Because murine perlecan domain V can be substituted with glycosaminoglycan side chains (37, 48), we subjected 10 μg of purified endorepellin to DEAE Sephacel chromatography. None of the purified endorepellin bound to the DEAE column under relatively low salt (NaCl, 150 mM) concentrations (Fig. 3*b*, *lane 3*) indicating that the human preparation did not contain glycosaminoglycan side chains. Interestingly, our construct behaves like the *Drosophila* perlecan domain V, which when expressed in the same 293-EBNA cells, migrates as a single band without any overt glycanation (49).

To test the biological properties of endorepellin, we utilized VEGF-induced migration of HUVEC to passages 4–8 (50). It is well established that the motility and vectorial migration of endothelial cells that coincidentally occur with invasion, are fundamental components of angiogenesis (33, 51, 52). When VEGF was used in the lower chamber, there was a complete suppression of HUVEC migration through the membrane at 1–10 μg ml^{-1} (12–120 nM) endorepellin (Fig. 3*c*). Interestingly, endorepellin was more active than recombinant endostatin purified from *Pichia pastoris* yeast cells. Subsequent dilution experiments revealed that endorepellin was fully active at 0.5 μg ml^{-1} (6 nM) (Fig. 3*d*), with a calculated IC_{50} of 2 nM (± 0.1, n = 11). In some preparations, endorepellin was active even at picomolar concentrations (not shown). These experiments were repeated several times with various preparations of endorepellin, and a marked suppression of HUVEC migration was consistently found. In contrast to endostatin, the migratory response was not dependent on the preincubation of the endothelial cells with endorepellin. In experiments where endorepellin was placed in the lower chambers of the invasion assay, we found similar inhibition of VEGF-induced migration (not shown).

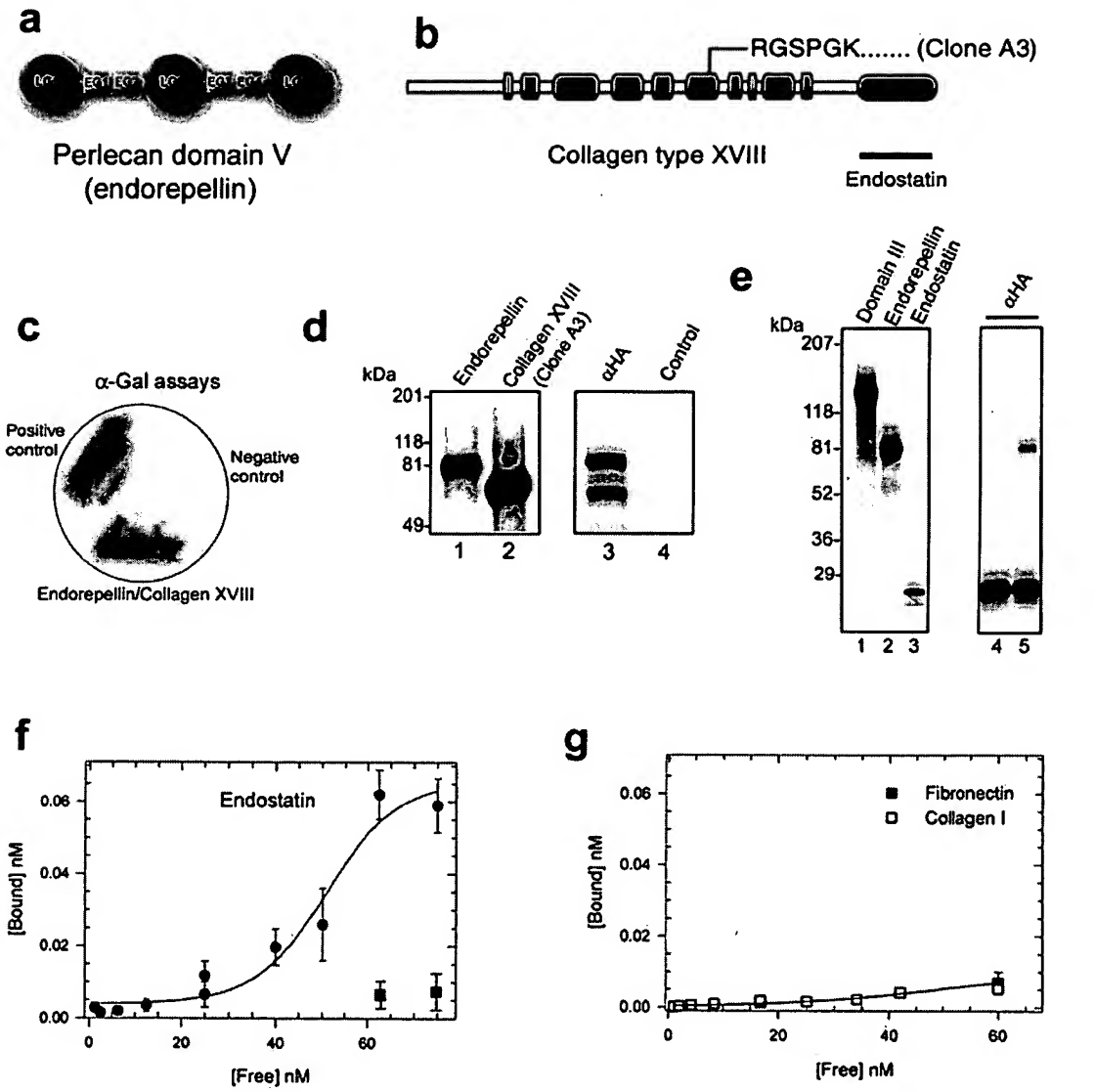

FIG. 1. **Perlecan domain V (endorepellin) binds to the anti-angiogenic factor endostatin.** *a*, schematic representation of human perlecan domain V/endorepellin which was used as a bait in the yeast two-hybrid screening. This domain consists of three laminin-type G (LG1–LG3) modules (*orange ovals*) separated by four EGF-like (EG1–EG4) modules (*blue rectangles*), in an arrangement highly conserved across species (2, 43). *b*, schematic representation of the human α chain of type XVIII collagen. The beginning of the clone A3 sequence (NCBI accession no. AF018082), which interacted with endorepellin, is shown in the *top margin*. The collagenous (triple-helical) and non-collagenous domains are indicated by *rods* and *blue boxes*, respectively. The C-terminal endostatin fragment is highlighted in *orange*. *c*, growth and α-galactosidase activity triggered by the interaction of endorepellin with collagen type XVIII fragment compared with the positive (p53 and T-antigen) and negative controls (lamin and T-antigen). *d*, co-immunoprecipitation of collagen XVIII (clone A3) and endorepellin following *in vitro* transcription/translation using [^{35}S]methionine as the labeled precursor. Endorepellin (*lane 1*) and the fragment of collagen XVIII (clone A3) (*lane 2*) were mixed in equimolar amounts and co-immunoprecipitated with either anti-HA (*lane 3*) or no antibody (*lane 4*). *e*, co-immunoprecipitation of endostatin with endorepellin. Domain III, a perlecan core protein domain used as a negative control (*lane 1*), endorepellin (*lane 2*), and endostatin (*lane 3*) were generated by *in vitro* transcription/translation using [^{35}S]methionine as the labeled precursor. Endostatin, which contains the HA tag at its C terminus, was mixed with either domain III (*lane 4*) or endorepellin (*lane 5*) and immunoprecipitated with anti-HA antibody. These experiments were repeated three times with comparable results. *f*, solid-phase binding assays of ^{125}I-endorepellin as the soluble ligand to endostatin-coated wells in the absence (●) or presence (■) of 25-molar excess of unlabeled endorepellin. The values represent the mean ± S.E. of quadruplicate determinations. *g*, solid-phase binding assays of soluble ^{125}I-endorepellin to either fibronectin (■) or collagen I (□) as the solid substrates. The recombinant proteins were coated onto Immulon wells at 10 μg ml^{-1} and then challenged with increasing concentrations of ^{125}I-endorepellin (~10^{18} cpm mol^{-1}) as indicated. The values represent the mean ± S.E. of quadruplicate determinations. These experiments were repeated twice with comparable results.

Next, we investigated whether the inhibition of HUVEC migration could lead to a decreased angiogenesis *in vivo*. Using the chorioallantoic membrane (CAM) assay, we discovered that endorepellin significantly reduced the angiogenic activity of VEGF (Fig. 4). In the presence of VEGF, the characteristic spoke wheel-like vessel formation was induced toward the sponge (Fig. 4*a*). In the presence of endorepellin (Fig. 4*b*), the vessel sprouts were markedly reduced to a level comparable to the negative control (Fig. 4*c*).

Next, we tested whether endorepellin could counteract the angiogenic stimuli of WiDr, a highly tumorigenic colon carcinoma cell line (53). Indeed, long term culture of capillary endothelial cells was originally obtained by culturing endothelial cells with media conditioned by malignant cells (54). This indicates that tumor cells express a large repertoire of growth-promoting factors that support endothelial cell survival and proliferation. We observed that the presence of endorepellin in the sponges harboring the colon carcinoma cells caused a

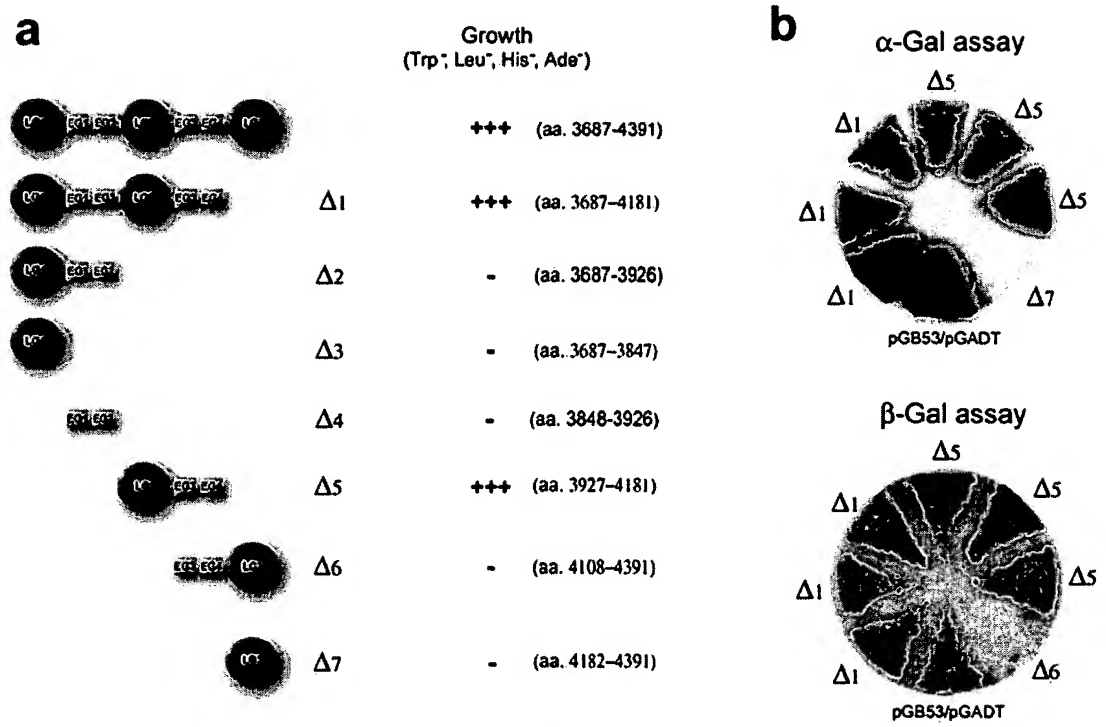

FIG. 2. **Endostatin specifically interacts with the LG2 module of endorepellin.** *a*, schematic representation of domain V and various deletion mutants. This domain consists of three laminin-type G (LG1–LG3) modules (*orange ovals*) separated by four EGF-like (EG1–EG4) modules (*blue rectangles*) in an arrangement highly conserved across species (2, 43). The growth is indicated by semi-quantitative assessment with maximal growth at +++. The numbers within parentheses designate the amino acid position based on the mature protein core. *b*, representative α- and β-galactosidase assays of various deletion mutants; pGB53/pGADT is the positive control.

marked suppression of the angiogenic process (Fig. 4*e*) as compared with the tumor cells themselves (Fig. 4*d*). Quantification of both sets of experiments using the NIH Image analysis software showed a 74 and 80% inhibition of vessel area around the sponges ($p < 0.001$) (Fig. 4, *f* and *g*, respectively).

To further investigate the role of endorepellin in *in vivo* angiogenesis, we performed Matrigel plug assays in *nu/nu* animals (40). To this end, we injected ~100 μl of Matrigel supplemented with FGF2 (10 ng/animal) and either BSA or endorepellin (12 μg/animal) into the dorsal subcutaneous regions of ten nu/nu mice. Mice were sacrificed 2 weeks after the injection and the skin removed to analyze the blood vessel formation. Inasmuch as the Matrigel plug is initially avascular, any vessels found within the plug must be, of necessity, new vessels (40). There were striking differences between control and endorepellin-treated samples. In the latter case, there was a marked inhibition of neovascularization around and within the Matrigel plug (Fig. 5*b*) as compared with the control samples (Fig. 5*a*). Microscopic examination showed marked ingrowths of new blood vessels in the control samples (Fig. 5*c*), but essentially little or no blood vessel formation in the presence of endorepellin (Fig. 5*d*). Quantification of the blood vessel density, as described above, again showed a marked (>75%) suppression of new blood vessels in the presence of endorepellin.

Finally, we tested endorepellin during HUVEC tube formation in a collagen matrix, a process thought to mimic morphogenesis (39). The results showed a capillary-like network formation in the control HUVECs (Fig. 5*e*), which was visible at 4 h, and remained constant for up to 24 h (not shown). In contrast, endorepellin caused a complete block of tube-like formation at concentrations similar to those used in the migration assays (Fig. 5*f*), whereas no significant effects were obtained with endostatin (Fig. 5*g*). Interestingly, endostatin was not capable of blocking the activity of endorepellin (Fig. 5*h*), suggesting that these two proteins possess distinct mechanisms of action (see below).

Collectively, our results indicate that endorepellin is a powerful blocker of angiogenesis and that its effects are long lasting.

Biological Effects of Endostatin/Endorepellin Interaction—To further investigate the biological significance of the interaction between endostatin and endorepellin, we performed several VEGF-induced HUVEC migration experiments in which the amount of endorepellin was kept constant while the amount of endostatin was proportionally increased. We chose two concentrations of endorepellin, 1.2 and 3.7 nM (100 and 300 ng ml^{-1}, respectively) that gave suboptimal and optimal inhibition of HUVEC migration. When endostatin and endorepellin were concurrently present, there was an overall inhibition of their activities (Fig. 6, *a* and *b*). By plotting the percentage of migrated cells derived from normalized data of five independent experiments, against the increasing molar ratios of endostatin/endorepellin, maximal neutralization was achieved at ~1:1 molar ratio. Thus, the combined effects of endostatin and endorepellin are not additive, but they may lead to an attenuation of their respective anti-angiogenic activities.

Specific Binding of Endorepellin to Endothelial Cell Surface—Next, we sought to determine whether endorepellin could specifically bind to the cell surface of HUVECs. We labeled endorepellin with ^{125}I to high specific activity (~10^{18} cpm mol^{-1}) and found the predominant 81-kDa band, with a small fraction of labeling going into a 25-kDa fragment (Fig. 7*a*). To establish the nature of this fragment, we transferred a similar preparation to polyvinylidene difluoride membrane and sequenced the N terminus. This confirmed that the 25-kDa fragment encompassed nearly all the LG3 module, with a specific cleavage between Asn-4196 and Asp-4197 (Fig. 7*a*). Covalent

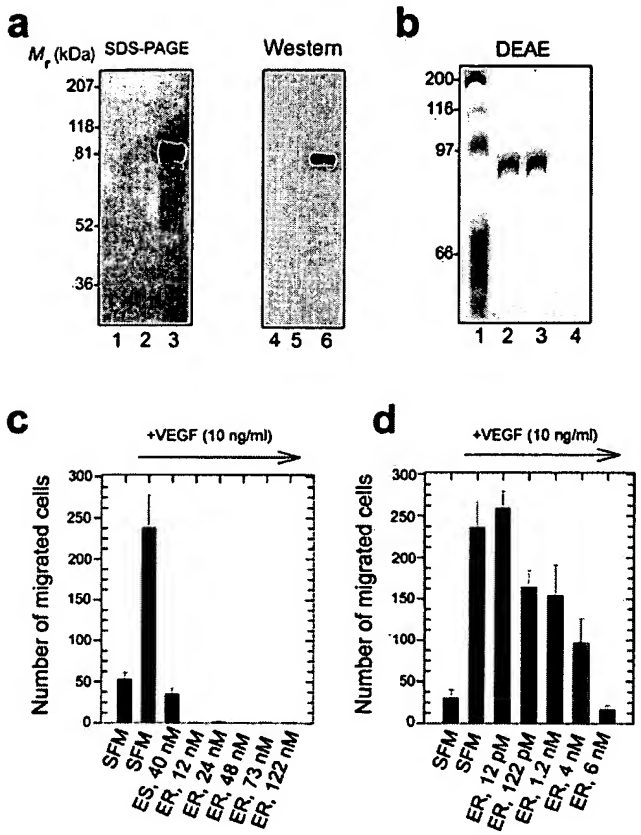

FIG. 3. **Recombinant endorepellin inhibits VEGF-mediated chemotactic migration of endothelial cells.** *a*, purification of endorepellin from media conditioned by 293-EBNA cells expressing the 81-kDa endorepellin tagged with His_6. Coomassie-stained SDS-PAGE (*left*) and Western immunoblotting with anti-His_6 antibody (*right*) of negative control media (*lanes 1* and *4*), flow through (*lanes 2* and *5*), and 250 mM imidazole eluate (*lanes 3* and *6*). *b*, Coomassie-stained SDS-PAGE of purified endorepellin following elution from a DEAE Sephacel chromatography. *Lane 1*, molecular weight rainbow markers; *lane 2*, starting material; *lane 3*, 150 mM NaCl eluate; *lane 4*, 2 M NaCl eluate. *c* and *d*, HUVEC migration assays through fibrillar collagen type I using 10 ng ml^{-1} VEGF as a chemotactic inducer with or without incubation with various concentrations of endostatin (*ES*) and endorepellin (*ER*) as indicated. SFM, serum-free medium. The values represent the mean ± S.E. of quadruplicate determinations. These experiments were repeated three times with comparable results.

affinity cross-linking experiments using a membrane-impermeable cross-linker (BS^3) revealed a major complex of very high M_r, which did not penetrate the 7.5% SDS-PAGE (Fig. 7*b*). Interestingly, the exogenously added LG3 module (the 25-kDa band in Fig. 7*b*) was not cross-linked to HUVEC surface proteins, suggesting that this part of endorepellin is dispensable for binding.

The binding of endorepellin was saturable in the range of 10–20 nM (Fig. 7*c*). Scatchard analysis (Fig. 7*d*) revealed a single receptor population consisting of ~3.6×10^5 sites $cell^{-1}$ with a K_d of 11 nM. Specificity of the binding was further demonstrated by the efficient displacement of the HUVEC-bound ^{125}I-endorepellin by increasing amounts of cold endorepellin (Fig. 7*e*), with IC_{50} of 27 nM, in good agreement with the binding isotherm shown above. These experiments were repeated three times with comparable results. Thus, we conclude that HUVECs possess a significant number of high affinity endorepellin binding sites.

High Affinity Binding Sites for Endorepellin on Various Tumor Cell Lines—Next, we wished to test whether endorepellin-binding sites could be present on cells other than HUVECs. To this end, we fused the coding region of endorepellin to that of the heat-stable human placental AP (55), a soluble marker that can be readily detected by chemiluminescence's reagents (42). We isolated several clones that expressed relatively high levels of endorepellin/AP chimeric protein (Fig. 8*a*) and quantitative analysis, using a standard curve based on AP activity, revealed that 10^5 clone 6 cells expressed ~4 μg ml^{-1} 48 h^{-1}. Immunoprecipitation studies using an anti-AP monoclonal antibody linked to agarose showed the predicted sizes of ~141 kDa for the AP and endorepellin/AP chimera, respectively (Fig. 8*b*). Notably, incubation of endorepellin/AP-conditioned media with HUVEC, MCF7, HT1080, and WiDr cells showed significant binding to the cell surface of all the cells, with the highest binding in WiDr followed by HUVEC, HT1080, and MCF7 (Fig. 8*c*). No binding was observed with AP alone (not shown), further indicating the specificity of the interaction between soluble endorepellin and the cell surface. We also tested A431 squamous carcinoma cells, but we could not block the endogenous AP even after 1–2 h incubation at 65 °C, indicating that these cells possess an endogenous heat-stable AP. To bypass this point, we performed binding studies of A431 and MCF7 (as a further control) cells using ^{125}I-endorepellin as the soluble ligand. The results showed a saturable binding for both A431 and MCF7 cells (Fig. 8, *d* and *f*). Scatchard analysis revealed a single receptor population for both A431 and MCF7 cells consisting of ~9 and ~7.5×10^4 sites $cell^{-1}$, with K_d values of 6.6 and 26 nM, respectively (Fig. 8, *e* and *g*). The lower number of receptors on the MCF7 as compared with the HUVECs is in full agreement with the binding studies shown in *panel c*. Notably, cross-linking experiments of A431 and MCF7 cells also showed the presence of high M_r complexes that did not penetrate the gel (not shown) suggesting that similar putative receptors are also present in these two cells.

Endorepellin Has Counter-adhesive Properties for Endothelial Cells—A number of bioactive fragments of extracellular matrix proteins exhibit counter-adhesive activity; that is, they disrupt cell-matrix interactions (52). It has been previously shown that domain V of perlecan, from either mouse or *Drosophila*, is adhesive for several cell lines when compared with fibronectin, but not for others (36, 49). To address this point, we tested whether endorepellin could mediate HUVEC adhesion. We found a complete lack of HUVEC adhesion to either endorepellin or BSA, in contrast to a robust adhesion to fibronectin or collagen type I (Table I). In competition experiments in which we challenged HUVECs with increasing amounts of endorepellin, we found a progressive inhibition of HUVEC attachment; within minutes the cells rounded up and began to detach in a dose-dependent manner (Fig. 9*a*). We performed several experiments on fibrillar collagen or plastic and, consistently, endorepellin prevented HUVEC binding to either substratum, with an IC_{50} of 5–20 nM. In contrast, endostatin did not show any interference with endothelial cell attachment to either fibronectin or collagen I (data not shown).

To verify that the anti-adhesive property of endorepellin was not just limited to endothelial cells, we tested HT1080 fibrosarcoma cells, which do not bind to murine domain V (36), and WiDr colon carcinoma cells (19). In both cases, endorepellin did not support adhesion (Table I). Moreover, specificity of endorepellin counter-adhesive properties was confirmed by the efficient displacement of HT1080 and WiDr attachment to fibronectin with increasing concentrations of recombinant endorepellin, with IC_{50} of 110 and 40 nM, respectively (Fig. 9*b*). In contrast, endostatin did not significantly affect the adhesion of either cell line (Fig. 9*c*). A summary of all the binding data, in which adhesion assays were performed using fibronectin and two concentrations of collagen type I, is provided in Table I. Interestingly, we found that endorepellin not only failed to

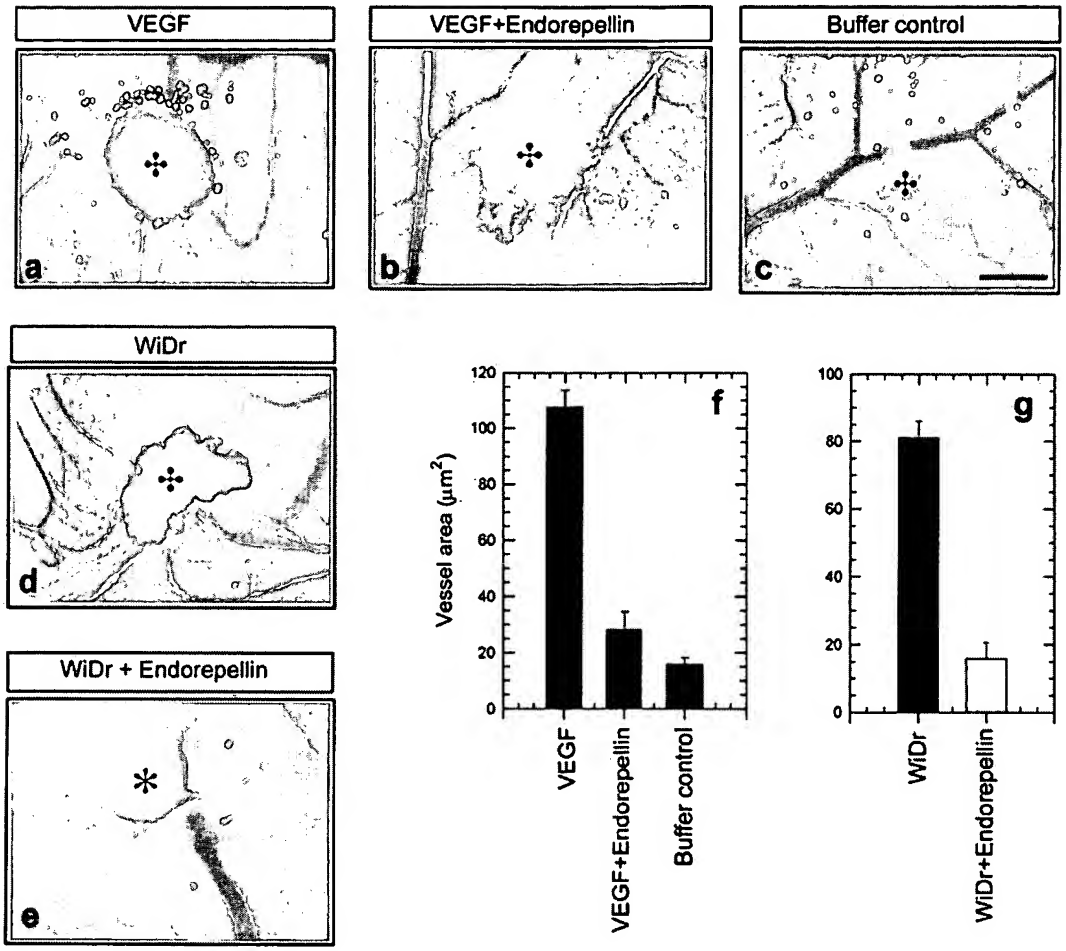

FIG. 4. **Recombinant endorepellin is a powerful anti-angiogenic factor *in vivo*.** *a–c*, CAM assays 3 days after the application of sponges (*asterisk*) containing VEGF (10 ng), VEGF (10 ng) + endorepellin (400 ng), or buffer alone. A total of 20 embryos were used for each experiment. *Scale bar*, 1 mm. *d* and *e*, CAM assays using sponges (*asterisk*) containing either 0.5×10^6 WiDr colon carcinoma cells alone or in combination with 3 μg of recombinant endorepellin. A total of 20 embryos were used for each experiment. These experiments were repeated three times with identical results. *f* and *g*, quantification of the CAM assays. To quantify the volume of the newly formed vessels, ten squares of ~500 μm^2 each were randomly selected around the sponge area. The mean vessel area was calculated using the NIH Image software program (version 1.61) using at least four embryos per experimental point. The values represent the mean ± S.E. of quadruplicate determinations. These experiments were repeated three times.

support adhesion for HUVECs, but also for most of the tumor cell lines tested, including HeLa, HT1080, WiDr, and M2 tumor cell lines. In contrast, A431 squamous carcinoma cells, which were previously shown to adhere to murine domain V, showed a mean attachment value of 52 ± 4% nearly identical to what has been previously obtained (36). We also found that MCF7 breast carcinoma cells had a similar (50 ± 7%) attachment value. Thus, endorepellin is a powerful anti-adhesive factor for endothelial cells and certain tumor cells, while it is partially adhesive for other tumor cell lines.

DISCUSSION

In an *in vivo* screening using the entire C-terminal domain V of human perlecan as bait, we discovered a strong interacting protein comprising the C terminus of human collagen type XVIII, including the anti-angiogenic factor endostatin (44). It has been previously shown, using a cell-free system, that perlecan proteoglycan binds to endostatin, presumably via the heparan sulfate chains (56, 57). We independently confirm these results and further show that a distinct subdomain of perlecan protein core specifically binds to endostatin. Using a battery of deletion mutants, the major binding site was mapped to the second laminin-like G domain of perlecan domain V. Because perlecan and type XVIII collagen/endostatin co-distribute in basement membranes (3, 22, 45, 46, 56), and because endostatin binds *in situ* to vascular basement membranes independently of heparan sulfate (58), we propose that domain V is a binding site for endostatin *in vivo*. Therefore, one outcome of these results, from a physiological point of view, would be that we have discovered an important interaction between the C terminus of perlecan and the C terminus of type XVIII collagen. This interaction could play a key role in the assembly of basement membranes and, perhaps, in the maintenance of their integrity.

Surprisingly, using HUVEC migration assays, we discovered that, while the interaction between endostatin and domain V counteracted their activities, perlecan domain V itself was a powerful anti-angiogenic factor, and hence we named it endorepellin. Endorepellin was active at nanomolar concentrations and was a potent inhibitor of angiogenesis in four independent assays commonly used to study angiogenesis: endothelial cell migration through fibrillar collagen, collagen-induced capillary-like formation, and growth of blood vessels in the CAM and Matrigel plug assays. The action of endorepellin was as strong as endostatin in inhibiting HUVEC migration, and in some experiments was even stronger than endostatin. Interestingly, endorepellin was also capable of counteracting

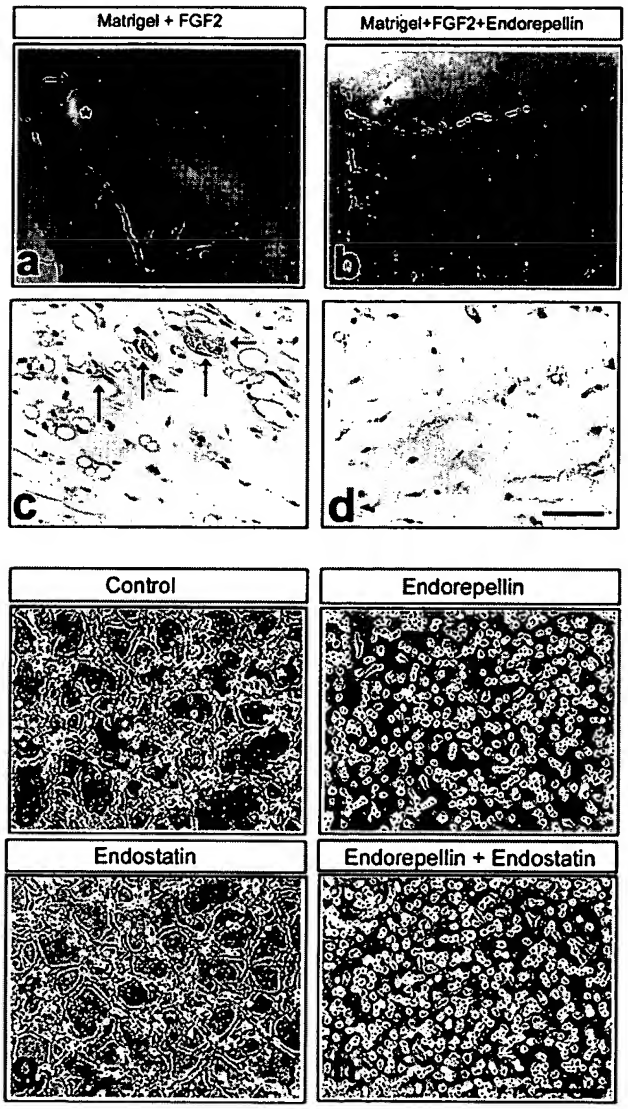

FIG. 5. **Endorepellin blocks blood vessel ingrowth in the Matrigel plug, and prevents endothelial tube formation induced by fibrillar collagen.** *a* and *b*, digital images of dorsal skin viewed from the inside, 2 weeks after subcutaneous injection of Matrigel supplemented with FGF2 and either BSA or endorepellin. Notice the decreased neovascularization around the Matrigel plug (*asterisk*) in the endorepellin-treated samples as compared with the control samples (*arrows*). *Scale bars*, 5 mm. *c* and *d*, photomicrographs of Matrigel plugs from either control or endorepellin-treated samples, respectively. The ingrowths of new blood vessels are markedly enhanced in the control samples (*arrows*), as compared with the endorepellin-treated samples. *Scale bars* = 200 μm. *e–h*, gallery of light micrographs capturing the production of HUVEC tube-like formation within a collagen type I matrix either alone or following the addition of endorepellin, endostatin, or both. Several concentrations of endorepellin and endostatin (50–150 nM) were tested. In this assay, 4×10^5 cells were incubated for 24 h, and pictures were taken at various intervals. The images shown are from the 4-h time point. The images at 24 h were identical to those obtained at 12 h (not shown), indicating that the effects of endorepellin are long lasting. These experiments were repeated three times with comparable results. *Scale bar*, 250 μm.

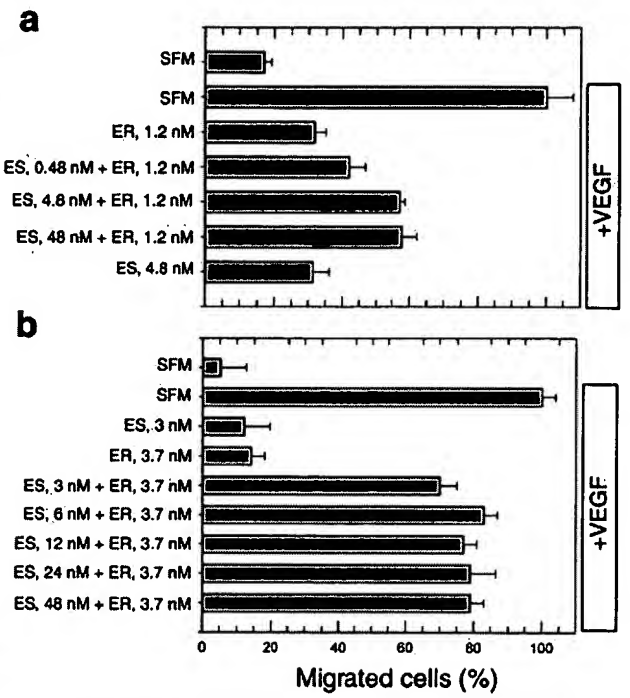

FIG. 6. **Biological consequences of endostatin/endorepellin interaction.** *a* and *b*, HUVEC migration assays through fibrillar collagen using 10 ng ml^{-1} VEGF as a chemotactic inducer and preincubation of HUVECs for 30 min with various concentrations of endostatin (*ES*), endorepellin (*ER*), or various combinations as indicated. The values are presented as the percentage of maximal migration induced by VEGF, arbitrarily set at 100%. *Panel a* shows the summary of three independent experiments run in quadruplicate, mean ± S.E. The values in *panel b* derive from an additional experiment run in quadruplicate, mean ± S.E. *SFM*, serum-free medium.

the angiogenic properties of WiDr colon carcinoma cells in the CAM assays. Notably, these cells synthesize large amounts of perlecan (19), which has been recently shown to bind FGF2 with affinities even higher than the endothelial cell perlecan (59). Thus, it is possible that endorepellin might act in a negative dominant fashion, at least in regard to the inhibition of capillary formation. We recently found that 293-EBNA cells expressing endorepellin do not form tumors in nude mice, in contrast to the wild-type cells,[2] suggesting that endorepellin might also play an anti-tumorigenic role *in vivo*.

We found a significant number (~3.6×10^5 $cell^{-1}$) of endorepellin binding sites on HUVECs, with a relatively high affinity constant (K_d = 11 nM). The specificity of binding was proved by the efficient displacement of the HUVEC-bound ^{125}I-endorepellin by increasing amounts of cold endorepellin, with an IC_{50} of 27 nM, in good agreement with the affinity constant mentioned above. The presence of putative endorepellin receptor(s) was further corroborated by the presence of high M_r complexes cross-linked to endorepellin. We also found high affinity binding sites on A431 and MCF7 tumor cells using radioligand binding assays similar to those used for HUVECs.

Endorepellin interfered with the adhesive properties of endothelial cells for various substrata, including fibronectin and fibrillar collagen, without directly binding to either protein matrix, and was also anti-adhesive for certain tumor cells derived from colon, neuroectoderm or mesenchyme. This is in agreement with previous studies showing anti-adhesive properties for perlecan in hematopoietic (30), mesangial (31), myoblastic (60), and smooth muscle (61) cells, and a role for perlecan in the suppression of growth and invasion in fibrosarcoma cells (62). However, while endorepellin inhibits tube formation and prevents cell adhesion to fibronectin and other substrata, monomeric endostatin does not. Thus, the two molecules may act via distinct mechanisms. We should point out, however, that oligomeric endostatin and the NC1 domain of collagen type XVIII have been recently shown to effectively inhibit tube morphogenesis (63), indicating that oligomerization is an im-

[2] M. Mongiat and R. V. Iozzo, unpublished observations.

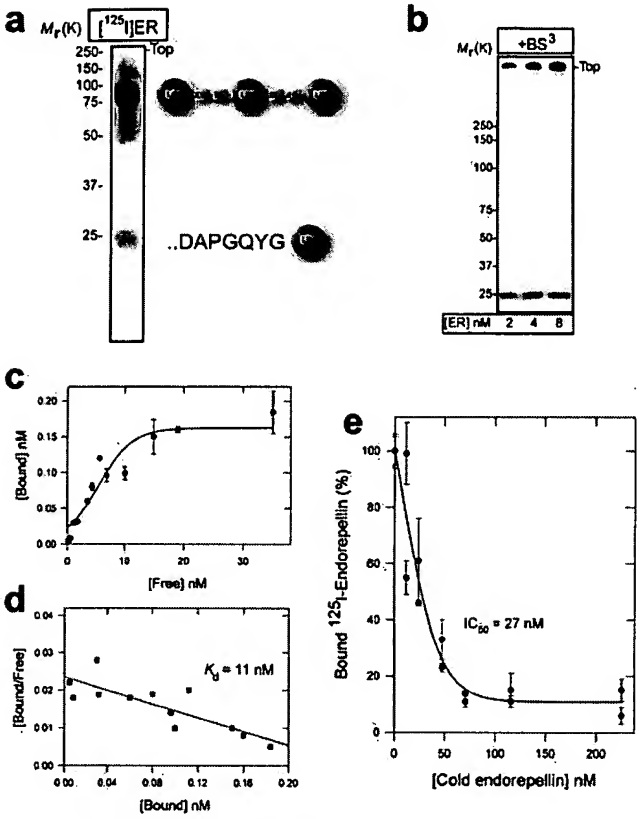

FIG. 7. **High affinity binding sites for endorepellin on endothelial cells.** *a*, autoradiography on a 10% SDS-PAGE of endorepellin (*ER*) labeled to high specific activity (~ 10^{18} cpm mol^{-1}) using Iodogen (Pierce). The autoradiograph was purposefully overexposed to show the minor contaminant band of ~25 kDa. About 15 pmol of the purified 25-kDa band were electroblotted onto a Problott-polyvinylidene difluoride membrane and microsequenced. The first N-terminal amino acid residue is Asp-4197, near the beginning of the LG3 module. The seven amino acid residues match perfectly to the human sequence of perlecan (7, 8). *b*, covalent affinity cross-linking. HUVECs were incubated with various concentrations of ^{125}I-endorepellin for 2 h as indicated in the *bottom*, and then incubated for 30 min with 2 mM BS^3, a membrane-impermeable cross-linker. The reaction was terminated with 1 M Tris, pH 7.5, the cross-linked material was separated on 7.5% SDS-PAGE, and visualized by autoradiography. Notice that endorepellin, but not the LG3 module, is complexed with high M_r material that does not penetrate the separating gel. *c*, saturation binding of ^{125}I-endorepellin on HUVECs. Confluent HUVECs in 24-well plates were incubated with increasing concentrations of ^{125}I-endorepellin for 2.5 h at 4 °C in M199 containing 0.1% BSA, washed several times, and extracted in the presence of protease inhibitors (41). Values represent the mean ± S.E. of three independent experiments run in triplicate. Nonspecific binding was subtracted from the observed values. *d*, Scatchard analysis of the data presented in *c*. Estimates of receptor affinity and total binding capacity were made with the Wizard program in the Sigma Plot 5.0 software package. These experiments were repeated three times with similar results. *e*, displacement of HUVEC bound ^{125}I-endorepellin by increasing amounts of cold endorepellin. The data represent the mean ± S.E. of two individual experiments run in triplicate. In these experiments, confluent HUVECs (~10^5 cells/dish) were incubated with ^{125}I-endorepellin (5 nM) plus increasing concentrations of recombinant unlabeled endorepellin, as indicated. The cells were incubated at 4 °C for 3 h, washed three times, extracted, and counted in total.

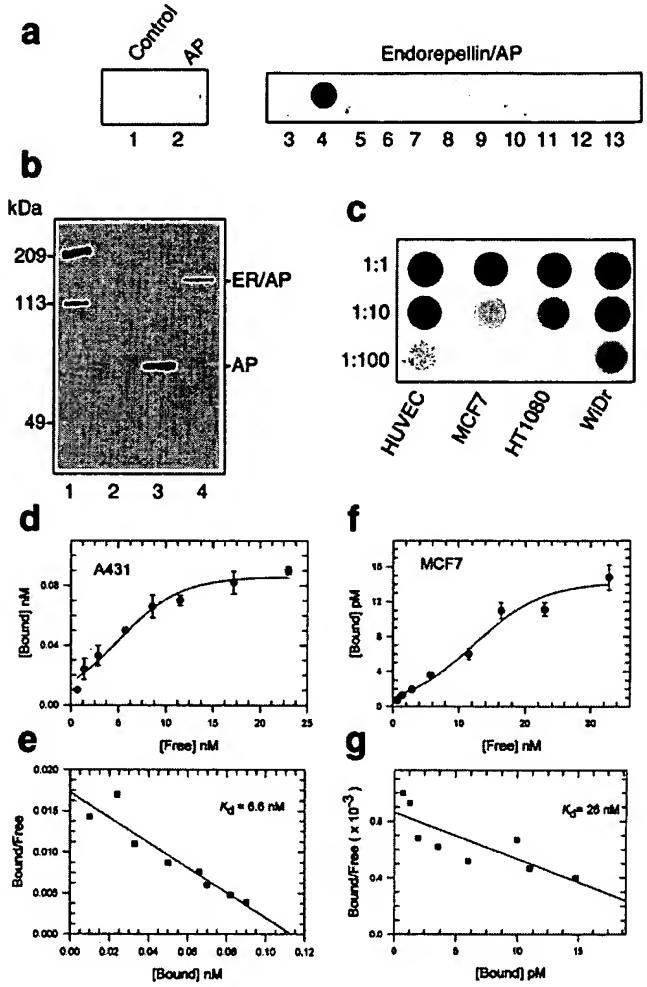

FIG. 8. **High affinity binding sites for endorepellin on various tumor cell lines.** *a*, generation of a cellular system secreting either AP or endorepellin/AP chimeric protein. *Lanes 1* and *2* represent conditioned media from either untransfected or AP-transfected 293-EBNA cells. *Lanes 3–13* represent media from positive and negative clones of 293-EBNA cells stably transfected with the endorepellin/AP construct. Conditioned media were incubated at 65 °C for 30 min to inactivate endogenous phosphatases, cooled on ice, and then mixed with CSPD substrate/chemiluminescent enhancer for 10 min (Great EscAPe™ SEAP system, Clontech), followed by exposure to x-ray film for 5–10 s. One of the strongest clone, (clone 6, *lane 4*) was amplified and used in the subsequent analyses in *b* and *c*. *b*, identification of AP alone or the chimeric endorepellin/AP protein following immunoprecipitation with a mouse monoclonal antibody (Clone 8B6, Sigma) against human placental AP linked to agarose beads. Coomassie Blue-stained 10% SDS-PAGE of 0.5 ml of conditioned media from control (*lane 2*), AP-secreting (*lane 3*), or endorepellin/AP-secreting (*lane 4*) 293-EBNA cells following incubation with 4 μl of antibody-agarose resin. Because 1 ml of settled resin binds at least 0.5 mg of human placental AP protein, we estimate that 10^5 clone 6 cells express ~4 μg ml^{-1} 48 h^{-1}. Molecular weight markers are in *lane 1*. *c*, binding of endorepellin/AP chimeric protein to various cells. Binding studies were performed using various cell lines (as indicated) incubated with 0.5 ml of serum-free media conditioned by expressing or control 293-EBNA cells for 48 h, using various dilutions as indicated in the *left margin*. After a 1.5-h incubation at 25 °C, the cells were washed six times, lysed in 1% Triton X-100, 20 mM Tris- HCl, pH 7.5, and processed for AP assays as above. *d* and *f*, saturation binding curves of ^{125}I-endorepellin on A431 squamous carcinoma (*d*) and MCF7 breast carcinoma (*f*) cells. Confluent cells in 24-well plates were incubated with increasing concentrations of ^{125}I-endorepellin for 2.5 h at 4 °C in M199 containing 0.1% BSA, washed several times and extracted in the presence of protease inhibitors (41). Values represent the mean ± S.E. of two independent experiments both run in quadruplicate. Nonspecific binding was subtracted from the observed values. *e* and *g*, Scatchard analyses of the data presented in *d* and *f*, respectively. Estimates of receptor affinity and total binding capacity were made with the Wizard program in the Sigma Plot 5.0 software package.

portant feature for their activity. In support of this concept, deletion of *cle-1* NC1 domain, the *Caenorhabditis elegans* homologue of mammalian collagen XVIII, causes defects in cell migration and axonal guidance (64). Both of these defects can be rescued by ectopic expression of the NC1 domain, known to trimerize *in vitro*, but not by the monomeric endostatin domain.

Notably, the potent inhibitory activity on endothelial cell

TABLE I

Binding of various cells to fibronectin, collagen type I, and endorepellin

The values are derived from adhesion assays to fibronectin, collagen type I, or endorepellin at the indicated concentrations, mean ± S.E. (n = 4). The numbers are related to the attachment to fibronectin arbitrarily set at 100%; that is, the OD values at 600 nm achieved in the plateau region of the curve, and those of collagen and endorepellin as percentage of the fibronectin plateau. About 80% of the total cells were attached in the plateau region of the fibronectin curve. In these experiments, 5×10^4 cells were seeded onto 96-well plates previously coated for 18 h at 4 °C with the various proteins at the indicated concentrations. After a 1-h incubation, the wells were extensively washed, and the number of adhered cells was estimated by a colorimetric assay.

Cell type	[Fibronectin] 50 nM	[Collagen I] 660 nM	[Collagen I] 50 nM	[Endorepellin] 50 nM
HUVEC, endothelial	100 (±5)	93 (±2)	104 (±5)	<5
A431, squamous carcinoma	100 (±6)	84 (±12)	72 (±6)	52 (±4)
HeLa, squamous carcinoma	100 (±10)	92 (±9)	ND[a]	<5
HT1080, fibrosarcoma	100 (±2)	100 (±10)	ND	<5
WiDr, colon carcinoma	100 (±6)	ND	ND	<5
MCF7, breast carcinoma	100 (±2)	182 (±2)	169 (±5)	50 (±7)
M2, mouse melanoma	100 (±5)	81 (±3)	77 (±3)	<5

[a] ND, not determined.

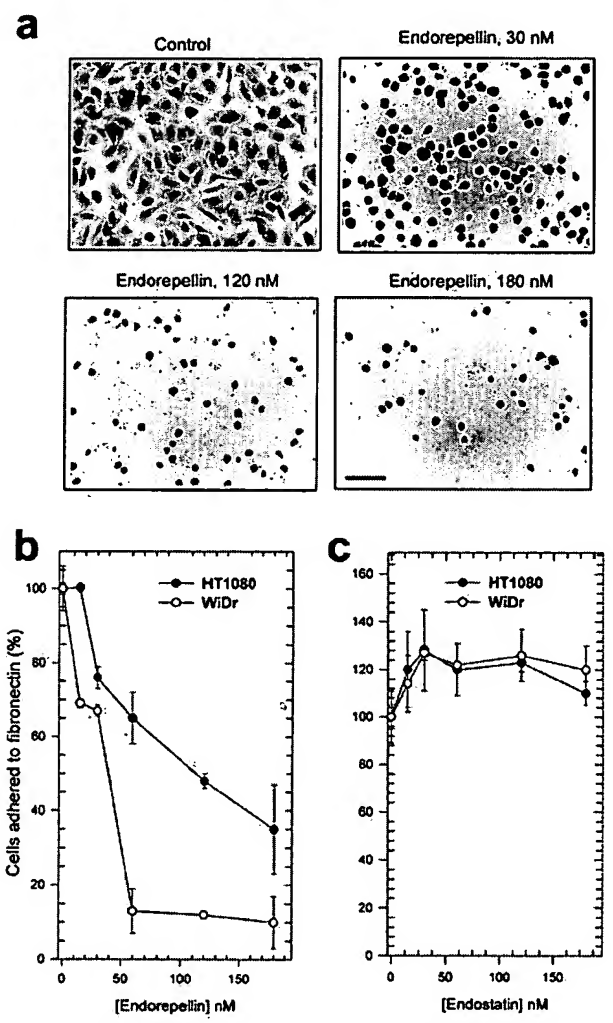

FIG. 9. **Endorepellin is counter-adhesive for endothelial, fibrosarcoma and colon carcinoma cells.** *a*, gallery of light micrographs of crystal violet-stained HUVECs adhered to fibronectin following incubation with endorepellin at the indicated concentrations. Briefly, the cells were trypsinized and plated onto fibronectin precoated (50 nM) wells in the presence of increasing concentrations of endorepellin or in the presence of phosphate-buffered saline (*control*). The cells were then incubated for 1 h, washed, and stained with crystal violet. After washing again, the cells were solubilized with Triton X-100, and the OD at 600 nm was determined. The adhesion assays were conducted in serum-free M199 medium. *Scale bar*, 100 μm. *b* and *c*, displacement of HT1080 ( ) and WiDr (○) cells from fibronectin-coated wells with increasing concentrations of either endorepellin or endostatin, respectively. The calculated IC_{50} values for HT1080 and WiDr were 110 and 40 nM, respectively. The values represent the mean ± S.E. (n = 4).

migration by endorepellin was neutralized by endostatin. Presumably, this occurs by the tight binding of endostatin to endorepellin that would alter the ability of endorepellin to interact with the cell surface. A logical extension of this hypothesis would be that this binding would also block the other activity (tube formation) of endorepellin. However, we observed that endostatin did not neutralize this activity. Migration of endothelial cells and tube-like formation are two different mechanisms that involve activation of different pathways (33, 65). The former occurs immediately after an angiogenic stimulus has taken place, whereas the latter involves the differentiation of the endothelial cells at the end of the angiogenic response. Thus, it is possible that the two proteins act on different receptors and that they activate similar or overlapping pathways during cell migration, but differ in the morphogenetic process of tube-like formation within a collagen matrix. It is also possible that endorepellin may bind to more than one receptor, each one involved in controlling different cellular mechanisms.

Powerful angiogenesis inhibitors are proteolytically processed forms of basement membrane collagens types IV, XV, and XVIII, the latter two being chondroitin and heparan sulfate proteoglycans, respectively (66). Moreover, proteolytic remodeling of the extracellular matrix can expose cryptic sites within collagen type IV that are required for angiogenesis *in vivo* (67), Thus, it is likely that perlecan might undergo a similar proteolytic processing *in vivo*, thereby liberating endorepellin through an endogenous processing mechanism common to most LG domains of laminin (43, 68, 69). The modular nature of perlecan protein core is particularly well suited for selective proteolysis (17, 66) and subsequent release of peptides with biological activity. There are several lines of evidence that support this scenario. First, in our 293-EBNA cells we detected a natural 25-kDa proteolytic cleavage product of endorepellin, which bound to the Ni-NTA column and was also reactive with the anti-His_6 antibody, indicating that it represented LG3. This was further confirmed by N-terminal sequencing analysis, which perfectly matched the seven amino acid residues starting with Asp-4197. Second, a similar band was previously shown to represent a proteolytic fragment of murine domain V generated by cleavage just before the beginning of LG3 (36, 37). This protease-sensitive region, which starts with the sequence DAPGQYG, is completely conserved between mouse (6) and human (7, 8), thus demonstrating that a specific cleavage of an Asn–Asp bond (at positions 3514–3515 and 4196–4197, for the mouse and human counterpart, respectively) had occurred near the N terminus of LG3. Mutational analysis indicated that Asp, but not Asn, is crucial for processing of mouse endorepellin (37), possibly by a specific, yet to be discovered, Asp-N endoproteinase. In our study the LG3 module failed to be cross-

linked to surface proteins, suggesting that this part of endorepellin is dispensable for surface binding. Third, an identical proteolytic fragment of ~25 kDa, cleaved at the same position as the mouse, was detected in the urine of patients with end-stage renal failure (70). This indicates that the LG3 module is present in the human serum at relatively high concentrations, since this LG3 was found at concentrations of ~10 mg liter^{-1} of urine (70). Fourth, we have recently discovered an additional proteolytic cleavage site (between Gly-3774 and Asp-3775) within the LG1 subdomain that leads to the release of almost the entire endorepellin lacking only the first 88 amino acid residues.[3] While this has not been proven to occur in tissues, it is plausible to take place because of the specificity of the cleavage site and the relatively high amounts of this fragment that we obtained after purification in which the mixture of protease inhibitors was suboptimal. Circulating forms of endorepellin may be involved in the homeostatic control of angiogenesis as previously proposed for endostatin, whose levels can reach 0.3 mg liter^{-1} of blood (56). We would like to put forward a provocative hypothesis, that is, it has been nearly three decades since it was shown that extracts of cartilage contain potent anti-angiogenic factors (71, 72), and because perlecan is highly expressed in cartilage, both during development and adulthood (9, 29, 73), endorepellin could conceivably be generated from the active remodeling of cartilage that occurs during normal aging, inflammation or any other condition that leads to cartilage turnover.

We do not yet know the precise mechanism of action of endorepellin. Two cell-surface proteins might be involved, either separately or in conjunction, namely, β_1 integrin and α-dystroglycan, both of which have been shown to interact with perlecan domain V (36, 75, 76). In the case of α-dystroglycan, perlecan domain V was the strongest ligand (K_d of 3 nM) and required LG1 and LG2 modules, whereas LG3 module by itself had much lower affinity (76). In agreement with these *in vitro* binding assays, perlecan and α-dystroglycan co-localize at the neuromuscular junctions (77, 78) where they may serve as cell-surface acceptors for acetylcholinesterase. Interaction between perlecan and α-dystroglycan, together with laminin, may also play a key role in the assembly of basement membranes during early development (74). Experiments are underway to address these important issues.

Recent experimental tests on tumor-bearing animals are encouraging because protein-based inhibitors, such as endostatin, have the following three major advantages. 1) They can reduce the tumors to a manageable size. 2) They do not induce resistance, and 3) their toxicity is low (66). Endorepellin is a novel natural inhibitor of angiogeneis, and its use in cancer therapy has additional advantages insofar as endorepellin may also exert an anti-adhesive action on certain tumor cells. Thus, we predict that if these protein-based agents are used in concert with traditional therapies, which target neoplastic cells directly, we may manage, or even cure, some forms of cancers that are currently incurable.

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Angiogenesis assays: Problems and pitfalls

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Abstract

Many studies of angiogenesis inducers and inhibitors rely on *in vitro* or *in vivo* models as indicators of efficacy. However, as valuable as these models are, there are limitations to each one of these. This overview describes some of the principal methods now in use and discusses the advantages and some of the limitations of these methods. It is suggested that multiple assays, involving both *in vitro* and *in vivo* assays, are at present the best way to minimize the problems inherent in any specific assay.

One of the major problems in angiogenesis research has been the difficulty of finding suitable methods for assessing the angiogenic response. In the past, the development of specific angiogenesis assays was dictated by the research problem under investigation. For example, the 96-well rapid screening assay for cytokinesis was developed in order to permit screening of hybridoma supernatants. The chick embryo total explant assay was designed to permit sequential photography during the angiogenic response to tumor cells. Transparent chamber methods were chosen to permit monitoring of the microcirculation. *In vitro* tests in general have been limited by the availability of suitable sources for endothelial cells, while *in vivo* assays have proven difficult to quantitate, limited in feasibility, and the test sites are not typical of the *in vivo* reality.

In a recent review, Rakesh Jain describes well what an optimal angiogenesis assay should achieve:

> *"(1) the release rate and the spatial and temporal concentration distribution of angiogenic factor(s)/inhibitor(s) should be known for generating the dose response curves; (2) if neoplastic cells are used as a source of angiogenic factors, they should be genetically well defined in terms of oncogene expression and production of growth factors (stimulators and inhibitors); (3) the assay should provide a quantitative measure of the structure of the new vasculature (e.g. vascular length, surface area, volume, number of vessels in the network, fractal dimensions of the network and extent of basement membrane;) (4) it should provide a quantitative measure of the functional characteristics of the new vasculature (e.g. endothelial cell migration rate, proliferation rate, canalization rate, blood flow rate, and vascular permeability; (5) there should be a clear distinction between newly formed and pre-existing host vessels; (6) tissue damage should be avoided, since it may lead to formation of new vessels; (7) any response seen in vitro should be confirmed in vivo; (8) such an assay should permit long-term and, if possible, noninvasive monitoring; and (9) it should be cost-effective, rapid, easy to use (routine), reproducible and reliable."* [1].

In vitro assays

Cell cultures

As we and others have pointed out, *in vitro* assays can be exceedingly useful in screening for specific functions (e.g. mitogen for vascular endothelial cells; inhibition of cytokine secretion; reduction in cell motility) [2–4]. These assays frequently do not translate into effects on angiogenesis *in vivo* because of the complex nature of *in vivo* angiogenesis. In all instances, *in vitro* screens can help identify optimal compounds or likely concentrations for efficacy, but they must be followed

by *in vivo* studies. *In vitro* assays that are readily quantifiable include matrix dissolution (metalloproteinase) assays, cell proliferation assays, cell motility (phagokinetic track; *in vitro* wounding) assays, chemotaxis (trans-membrane) assays, and tube formation assays (in collagen gels, on Matrigel), each reflecting one particular step involving endothelial cells during their response to angiogenic stimulation.

A serious problem in *in vitro* assays utilizing endothelial cells is the difficulty in obtaining adequate numbers of primary isolates [5,6]. Thus, almost all assays involve cells which have been expanded *in vitro*. There are significant changes during prolonged culture, including alterations in activation state, karyotype, expression of cell surface antigens and growth properties. The last of these, especially, presents a significant impediment to using these cells as a model for *in vivo* reactions because endothelial cells are normally in a quiescent state. Endothelial cells are among the cells with the lowest proliferative rate *in vivo* except upon activation or in special circumstances such as is seen in the ovarian reproductive cycle or pregnancy [7].

Another problem, now gradually gaining recognition, is the fact that all endothelial cells are not alike, and that the response to growth factors and inhibitors varies with the source of these endothelial cells. Microvascular endothelial cells, the primary responding cells during angiogenesis, differ for different organs, within different blood vessels of those organs, and even within individual blood vessels [8–11]. For example, brain endothelial cells express multidrug resistance genes, manifest brain-specific antigens on their cell surface, synthesize gamma glutamyl transferase and show a highly distinct, characteristic specialized morphology [12]. Primitive and tumor-induced blood vessels, on the other hand, show marked upregulation of vascular cell adhesion molecules and specific integrin receptors, as well as several not yet fully characterized cell surface antigens. Moreover, there is heterogeneity even among tumor-associated endothelial cells, as can be readily demonstrated by experiments which show that different tumors show a highly selective capacity to adhere to organ and site-specific blood vessels [10,11,13–15]. Moreover, different endothelial cells produce different cytokines (including both inhibitors and inducers of angiogenesis), thus introducing yet more complexity into the assay systems. *In vitro* model systems that fail to take this marked diversity among endothelial cells into account are likely to miss many of the fine points underlying the *in vivo* angiogenic response.

One of the most faithful cell culture models for angiogenesis is the ability of endothelial cells to form three-dimensional tube-like structures when placed on matrix components such as collagen or Matrigel [15,16]. Here too there is marked heterogeneity, for endothelial cells differ greatly in their ability to form such structures, and other cell types generate tube-like structures that cannot be distinguished from endothelial cell tubes without ultrastructural confirmation.

There are two aspects of cell culture assays involving endothelial cells that are readily apparent but frequently overlooked. The first, especially important in testing anti-angiogenic factors, is that endothelial cells are difficult to maintain and hence readily inhibited or killed, although such endothelial cells are rugged and long-lived *in vivo*. The slightest change in pH or osmolarity is cytotoxic to endothelial cells *in vitro*. Thus many agents may prove to have efficacy in preventing endothelial cell growth, migration or structural rearrangement *in vitro* for reasons that have no relevancy to angiogenesis *in vivo*. Secondly, there are numerous cytotoxic agents and growth factors that are not specific to endothelial cells at all, and thus are unlikely to have selective effects on angiogenesis *in vivo*.

Organ cultures

The aortic ring assay. A three-dimensional *in vitro* assay which permits more complex interactions to occur is the aortic ring assay, in which an entire aortic segment is analyzed for response *in vitro* [17]. This organ-culture type system has the advantage of including effects mediated by non-endothelial cells subjacent to the vascular endothelium. Although difficult to evaluate and quantitate, the effects seen in this system sometimes are quite different from the effects seen when endothelium is used as the sole source of cells.

On the other hand, the aortic ring organ-culture system has disadvantages that are hard to overcome. Quantitation is exceedingly difficult, growth requirements differ between the explant and the cell outgrowth, serum-free cultures are only marginally successful, and, although the cell outgrowth may be of microvascular origin, the model as a whole is only mildly representative of the microvascular organ environment encountered during angiogenic reactions induced by tumors or inflammatory mediators.

The chick aortic arch assay. A recent new model, adapted from the aortic ring assay, is the chick

embryonic aortic arch assay, which overcomes some of these problems [18]. Embryonic aortic arch cultures readily grow in the absence of serum, and the phenotype of the embryonic endothelial cells more closely resembles microvascular or activated endothelial cells associated with angiogenic reactions than does that of adult aortic endothelial cells. The model, however, has only recently been described and more details are needed before its validity can be fully evaluated.

Embryo cultures

Methods for culture of whole avian or mammalian embryos have had only limited application in assessing angiogenesis, primarily because these explanted embryos do not survive for more than a few days. The principal use for mammalian and avian whole embryo cultures has been to study short-term effects of agents or of transgene expression, with the focus on vasculogenesis and organ formation [19].

An exception is the use of explanted chick embryos for study of angiogenic responses on the chorioallantoic membrane (CAM) [20]. Chick embryos can be explanted after 72 h incubation, i.e. during early stages of organogenesis, and maintained *in vitro* for an additional 8–12 days. The CAM forms within 2–3 days after explantation, leaving adequate time for assessing subsequent angiogenic reactions. The *in vitro* system has the advantage over *in ovo* procedures in that it permits continuous monitoring and photographic recording of the induced response. Additionally, multiple grafts can be placed on the same membrane, permitting the comparison of experimental and control grafts in the same culture.

Much caution needs to be exercised, however, when evaluating CAM grafts, be they *in ovo* or *in vitro*. The CAM is itself a rapidly growing organ with an expanding vasculature, which may obscure any pro-angiogenic effect. Any agent that causes local edema will appear to be anti-angiogenic at the test site. Any agent that promotes vasodilation will cause an apparent increase in blood vessels by making previously invisible small capillaries visible under the low power magnification normally used to observe CAM angiogenesis. Also, there are significant differences between the physiological requirements and functions of the avian extra-embryonic vasculature and those of the adult vasculature of humans and other mammalian species.

In vivo **assays**

The mouse corneal angiogenesis assay

When first described for the rabbit in 1974, the assay was immediately recognized as an almost ideal test system for observing neovascularization [21]. When angiogenesis-inducing factors or cells are introduced into a pocket made in the cornea at some distance from the limbus, the cornea, being itself avascular, becomes vascularized from the limbal vasculature. The method was adapted for use in mice, in order to take advantage of inbred strains, transgenic animals, and a rich background of information and reagents for this species, as well as to reduce cost and increase the number of tests that can be performed [22,23]. Subsequently the method has also been used in rats and guinea pigs. The corneal assay is considered by many to be the gold standard for tests of angiogenesis. It clearly measures only new vessels, the reaction can be monitored by inspection, and the assay lends itself well to the testing of augmenters and inhibitors of angiogenesis administered orally, systemically or topically.

There are some problems with the assay, however, that need to be considered. The very fact that the cornea is avascular makes it atypical: Angiogenesis *in vivo* does not normally occur in avascular regions. The corneal pocket itself is exempt from the many blood-borne factors that can influence endothelial cell maintenance or survival. Vessels entering the cornea grow almost two-dimensionally at the interface between the epithelium and the underlying stromal cells.

Sustained release formulations have been used to provide prolonged administration of substances introduced into the cornea [24,25]. However, many of these formulations are themselves irritating, leading to inflammatory reactions. Additionally, release kinetics within the cornea are not the same as in solution, and there tends to be an initial burst before release becomes linear. The introduction of cells and test substances in polyvinyl sponges avoids or reduces the use of sustained release polymers.

The intradermal angiogenesis assay

The intradermal angiogenesis assay was originally developed for studies involving the injection of allogeneic lymphocytes (normal lymphocyte transfer reaction) to measure effects of foreign lymphocytes on tumor growth within the dermis [26]. The vascular

changes include initial vasodilation followed by the induction and subsequent marked divarication of new blood vessels.

This assay requires critical controls, and the experimental design must include blinded observers, inasmuch as the enumeration of new blood vessels and branch points, key to quantitation of this assay, is subject to experimental bias. Automated image analysis methods have improved quantitation, but bias is still possible because preparation of the skin and exposure of the implant site are subject to variation.

The Matrigel plug assay

Matrigel, a complex, rich-in-laminin product of EHS tumors, has the property of being liquid at 4°C but semi-solid at 37°C. Thus it can serve as a vehicle for cells or test compounds, which are suspended in the liquid, and then injected subcutaneously into test animals where the Matrigel solidifies [27]. Growth factors such as VEGF or bFGF, or tumor cells, suspended in Matrigel, evoke an angiogenic reaction over the next days or weeks. Assessment of angiogenesis can be made by determining the extent of vessel ingrowth into the Matrigel plug. Alternatively, total hemoglobin content can be determined, thus measuring the blood content within the plug. As is the case of the cornea assay, drugs or other test substances can be introduced orally or systemically, and the angiogenic reaction in the Matrigel plug determined. Inasmuch as the Matrigel plug is initially avascular, any vessels found within the plug must, of necessity, be new vessels.

Unfortunately, there is considerable variability in the assay, largely because it is difficult to generate identical three-dimensional plugs even though the total Matrigel volume is kept constant. Histological analysis is time-consuming, and enumeration of blood vessels in sections is difficult. The hemoglobin assay, on the other hand, cannot distinguish between blood in capillaries and blood in sinuses or larger vessels.

Recently we have developed a two-stage Matrigel assay in which a sponge containing growth factors or tumor cells, or a tumor fragment, is implanted surgically into a Matrigel plug previously generated by subcutaneous injection of pure Matrigel (unpublished observations). In this system, angiogenesis is directional, and the new blood vessels can be visualized in whole mounts by injecting fluorescinated high molecular weight dextran intravenously a few minutes before removing and fixing the Matrigel plug. This modification of the Matrigel plug assay has increased sensitivity and measures angiogenesis more directly, but is more difficult to quantitate. Confocal microscopic examination may be able to improve the quantitative aspects of the Matrigel/sponge assay.

Chamber assays

Although historically one of the oldest methods of observing angiogenesis *in vivo*, the assay, which typically consists of a transparent chamber containing cells, tissues or sponges, lends itself best for continuous monitoring of the progressive changes occurring in the vasculature surrounding the angiogenic stimulus [28–30]. The assay is designed to permit *in situ* microscopic observations, and is the optimal method for measuring physiological effects such as perturbations in blood flow.

Both the initial placement of the chamber and the subsequent observations are difficult to carry out and time-consuming, and therefore data can be obtained on relatively few animals. It is important to recognize, however, that the surgical procedures and the physical attributes of the chambers are likely to induce vascular effects related to wound healing that are superimposed on the effects of the test cells or substances themselves.

The chick chorio-allantoic membrane assay

This assay, already described above under whole embryo culture, has more generally been carried out *in ovo* rather than *in vitro*. The CAM assay carried out in eggs is relatively simple when compared to the organ culture method discussed previously. Eggs are preincubated, an opening is made in the shell and shell membrane, and a graft, sponge, filter or sustained-release membranes are placed on the CAM. Unlike the chick explant method, however, monitoring of the test area is difficult, and reliance must almost entirely be placed on examination of the experimental site at the end of a prescribed period of incubation.

Although much reliance has been placed on this classical assay, there are several unfortunate artifacts that make validation difficult. Any irritant, including shell dust generated when making a window in the shell, and any sliver of shell membrane that may protrude and touch the CAM, will cause an angiogenic reaction. The membrane is also extremely sensitive to changes in oxygen tension, and thus sealing of the opening is critical. Monitoring of the angiogenic reaction by

reopening the seal may itself cause alterations in the response, and complete resealing of the opening in the shell is essential.

Concluding comments

This review is of necessity incomplete, for there are many modifications of each of the methods discussed. The field of angiogenesis research is expanding rapidly and thus the assessment of the angiogenic response becomes ever more important. Awareness of the problems and pitfalls in performing the various assays will lead to improvements in interpretation of results obtained with these assays and to the development of new assays to augment or replace existing ones. For the moment at least it seems that *in vitro* tests are better conducted with more than one type of endothelial cell, with more than one cell type, and with evaluation of more than one component of the angiogenic reaction. For validation, it will be essential to use *in vivo* assays, and as yet no single assay seems adequate. In selecting assays it will always be necessary to keep in mind the desired endpoint, whether this be inhibition of inflammatory reactions, acceleration of wound healing or reduction of tumor growth and metastasis.

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